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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 September 2003 (04.09.2003)

PCT

(10) International Publication Number
WO 03/072072 A1

(51) International Patent Classification[7]: A61K 7/021, 7/42, 38/22

(21) International Application Number: PCT/AU03/00230

(22) International Filing Date: 25 February 2003 (25.02.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/359,735 26 February 2002 (26.02.2002) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/072072 A1

(54) Title: METHOD OF STIMULATION OF MELANIN PRODUCTION AND INDUCTION OF SKIN TANNING

(57) Abstract: A method of stimulating the production of melanin by the pigment-producing cells (keratinocytes and/or melanocytes) of the skin, in particular for the induction of skin tanning in humans, comprises administering alpha-MSH or an alpha-MSH analogue and exposing the skin to ultraviolet irradiation.

METHOD OF STIMULATION OF MELANIN PRODUCTION AND INDUCTION OF SKIN TANNING.

Partial funding for the research leading to the present invention was received from the National Institute of Health. Accordingly, the Government of the United States of America retains certain rights in the invention described herein.

FIELD OF THE INVENTION

The present invention relates broadly to a method of stimulating the production of melanins by the pigment-producing cells (keratinocytes and/or melanocytes) of the skin, and in particular to a method for the induction of skin tanning in humans.

BACKGROUND OF THE INVENTION

The melanocortins (also referred to as melanotropin) include a family of peptide hormones that induce pigmentation by interaction with melanocortin 1 receptors (MC1R) in the epidermis[1]. The primary pigmentary hormone that is released from the pars intermedia of the pituitary gland in some non-human animals, and from UV-B exposed keratinocytes in human skin, is alpha Melanocyte Stimulating Hormone (alpha-MSH)[1]. This 13 amino acid peptide binds to MC1R to induce cyclic AMP-mediated signal transduction leading to the synthesis of melanin polymers from DOPA precursors[1]. Two type of melanins can be expressed in humans. The brownish - black pigment eumelanin is believed to convey protection from sun damage, whereas the reddish, sulfur-containing pigment, pheomelanin is often expressed in light-skinned human populations that report a poor tanning response to sunlight[2]. These poorly-tanning, easily-burning populations, termed Type 1-2 by Fitzpatrick scale[3], may possess defects in the MC1R gene[4], and are generally thought to be at a greater risk of developing skin cancers[5,6].

It has previously been disclosed that a super-potent derivative of alpha-MSH, melanotan-1, (Nle^4-D-Phe^7-alpha MSH), can induce tanning in human volunteers with such poorly-tanning skin types, but especially in subjects with easily tanning skin types, (Fitzpatrick scale 3-4)[7]. Melanotan-1 (MT-1), contains two amino acid substitutions and is approximately 100 to 1,000-fold more potent

than the native hormone at inducing pigmentation in experimental systems such as the frog skin bioassay[8] or in cultured human keratinocytes[8]. In humans, MT-1 primarily induces eumelanin synthesis in the skin in concert with it's tanning effect[9]. Although melanotropins have been postulated to effect immunologic changes[10-12], all of the prior trials reported only minimal side effects such as facial flushing and transient GI upset, unless doses greater than those needed for tanning were administered[13].

US Patent No. 4,457,864 (issued July 3, 1984), discloses analogues of alpha-MSH, including Nle^4-D-Phe^7-alpha MSH. Cyclic analogues of alpha-MSH are disclosed in US Patent No. 4,485,039 (issued November 27, 1984). The use of these and other analogues of alpha-MSH for stimulating the production of melanin by integumental melanocytes is disclosed in Australian Patent No. 597630 (dated January 23, 1987) and US Patents Nos. 4,866,038 (issued September 12, 1989), 4,918,055 (issued April 17, 1990) and 5,049,547 (issued September 17, 1991). Australian Patent No. 618733 (dated May 20, 1988), and US Patents Nos. 5,674,839 (issued October 7, 1997) and 5,714,576 (issued February 3, 1998) disclose further linear and cyclic alpha-MSH fragment analogues, and the use of these biologically-active analogues in stimulating melanocytes. The contents of all these published Australian and US patents are incorporated herein by reference (see also refs. [25,26]).

All of the previously-reported clinical trials with MT-1 were performed in human volunteers who were instructed to avoid sunlight and use sunscreens with an SPF of 30 to apply to all sun-exposed skin sites[7,9,13]. Thus, the effect in humans of MT-1 when combined with either sunlight or simulated UV radiation, has not been tested or reported otherwise.

In work leading to the present invention, the inventors have carried out clinical trials in human volunteers with MT-1 combined with either direct sunlight, or with small doses of UV-B radiation delivered from a solar simulator. The intent of these studies was to examine the effect of MT-1 on skin tanning in humans, and in particular, whether there was any evidence of additive pigmentation or an alteration in the response of skin to UV-B, measured by the presence of sunburn cells. In addition, a subset of patients receiving MT-1 underwent detailed analysis of 17 different B- and T-lymphocyte sub-populations to evaluate the effect of MT-1 on immunologic status.

As a result of these clinical trials, the inventors have discovered that the combined use of a melanotropic peptide such as MT-1 and UV radiation results in unexpected levels of skin tanning and

prolonged retention of pigmentation. Accordingly, the methods of the present invention enable enhanced skin pigmentation from sunlight exposure, reduction in the amount of sunlight exposure required for visually-apparent skin tanning, safe acceleration of the production of sun-protective skin tanning, and reduction of sun-induced skin damage by rapid induction of long-lasting eumelanin expression in sun-exposed areas.

SUMMARY OF THE INVENTION

Bibliographic details of the publications referred to in this specification by reference number are collected at the end of the specification.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications, the invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

In one aspect, the present invention provides a method for the stimulation of integumental melanocytes in a mammal, which comprises the steps of:

(i) administering to said mammal an amount of alpha-MSH or an alpha-MSH analogue effective to stimulate melanocytes in the skin or other epidermal tissue; and

(ii) exposing said skin or other epidermal tissue to ultraviolet (UV) irradiation.

In another aspect, the present invention provides a method for stimulating melanin production in a mammal, which comprises the steps of:

(i) administering to said mammal an amount of alpha-MSH or an alpha-MSH analogue effective to stimulate melanin production in the skin or other epidermal tissue; and

(ii) exposing said skin or other epidermal tissue to ultraviolet (UV) irradiation.

In yet another aspect, the present invention provides a method for inducing tanning in a mammal, which comprises the steps of:

(i) administering to said mammal an amount of alpha-MSH or an alpha-MSH analogue effective to induce tanning in the skin or other epidermal tissue; and

(ii) exposing said skin or other epidermal tissue to ultraviolet (UV) irradiation.

The present invention further extends to the use of alpha-MSH or an alpha-MSH analogue in a method for the stimulation of integumental melanocytes in a mammal, more particularly for stimulating melanin production, and even more particularly for inducing tanning in a mammal. In this aspect, the invention extends in particular to the use of alpha-MSH or an alpha-MSH analogue in a method for inducing skin tanning in a human.

DETAILED DESCRIPTION OF THE INVENTION

As described above, the present invention provides a method for the stimulation of integumental melanocytes in a mammal, which comprises the steps of:

(i) administering to said mammal an amount of alpha-MSH or an alpha-MSH analogue effective to stimulate melanocytes in the skin or other epidermal tissue; and

(ii) exposing said skin or other epidermal tissue to ultraviolet (UV) irradiation.

The present invention also extends to a method for stimulating melanin production in a mammal. Preferably, the mammal is a human, however the methods of the present invention also extend to other mammals in which increased melanin production may be desired, for example to change coat (hair) coloration. In particular, the invention relates to a method for inducing tanning in a mammal, more particularly, for inducing skin tanning in a human.

The step of exposing the skin or other epidermal tissue to UV irradiation may be carried out simultaneously with, or subsequent, to the step of administering the alpha-MSH or alpha-MSH analogue to the skin or other epidermal tissue. Preferably, the step of exposing to UV irradiation is carried out subsequent to administration of the alpha-MSH or alpha-MSH analogue.

The step of exposure to UV irradiation may be performed either by exposure to artificial UV-B and/or

UV-A irradiation from a solar simulator or similar UV source, or preferably, by exposure to natural sunlight. Preferably, the UV irradiation consists of or comprises UV-B irradiation.

Alpha-MSH analogues suitable for use in the method of the present invention include those disclosed in US Patents Nos. 4,457,864, 4,485,039, 4,866,038, 4,918,055, 5,049,547, 5,674,839 and 5,714,576 and Australian Patents Nos. 597630 and 618733, and the disclosure of each of these patent documents is incorporated herein by reference (see also refs. [25, 26]).

In its broadest aspects, the present invention extends to the use of any of these alpha-MSH analogues. These analogues may be synthesised according to the procedures set out in these patent documents or other references, or according to methods used in preparing synthetic alpha-MSH which are well-known to persons skilled in this art, for example, by solid phase peptide synthesis.

Suitable alpha-MSH analogues for use in accordance with the present invention include compounds of the formula:

R_1-W-X-Y-Z-R_2

wherein

R_1 is selected from the group consisting of Ac-Gly-, Ac-Met-Glu-, Ac-Nle-Glu-, and Ac-Tyr-Glu-;

W is selected from the group consisting of –His- and –D-His-;

X is selected from the group consisting of –Phe-, -D-Phe-, -Tyr-, -D-Tyr-, -(pNO_2)D-Phe^7-;

Y is selected from the group consisting of –Arg- and –D-Arg-;

Z is selected from the group consisting of –Trp- and –D-Trp-; and

R_2 is selected from the group consisting of –NH_2; -Gly-NH_2; and –Gly-Lys-NH_2.

As used hereinabove and below, Ala = alanine, Arg = arginine, Glu = glutamic acid, Gly = glycine, His = histidine, Lys = lysine, Met = methionine, Nle = norleucine, Phe = phenylalanine, (pNO_2)Phe = paranitrophenylalanine, Plg = phenylglycine, Pro = proline, Ser = serine, Trp = tryptophan, TrpFor = N^{1}- formyl-tryptophan, Tyr = tyrosine, Val = valine. All peptides are written with the acyl-terminal end at the left and the amino terminal end to the right; the prefix "D" before an amino acid designates the D-isomer configuration, and unless specifically designated otherwise, all amino acids are in the L-isomer configuration.

Compounds suitable for use in the present invention include:

[D-Phe^{7}]-alpha-MSH
[Nle^{4}, D-Phe^{7}]-alpha-MSH
[D-Ser^{1}, D-Phe^{7}]-alpha-MSH
[D-Tyr^{2}, D-Phe^{7}]-alpha-MSH
[D-Ser^{3}, D-Phe^{7}]-alpha-MSH
[D-Met^{4}, D-Phe^{7}]-alpha-MSH
[D-Glu^{5}, D-Phe^{7}]-alpha-MSH
[D-His^{6}, D-Phe^{7}]-alpha-MSH
[D-Phe^{7}, D-Arg^{8}]-alpha-MSH
[D-Phe^{7}, D-Trp^{9}]-alpha-MSH
[D-Phe^{7}, D-Lys^{11}]-alpha-MSH
[D-Phe-^{7}, D-Pro^{12}]-alpha-MSH
[D-Phe^{7}, D-Val^{13}]-alpha-MSH
[D-Ser^{1}, Nle^{4}, D-Phe^{7}]-alpha-MSH
[D-Tyr^{2}, Nle^{4}, D-Phe^{7}]-alpha-MSH
[D-Ser^{3}, Nle^{4}, D-Phe^{7}]-alpha-MSH
[Nle^{4}, D-Glu^{5},D-Phe^{7}]-alpha-MSH
[Nle^{4}, D-His^{6}, D-Phe^{7}]-alpha-MSH
[Nle^{4}, D-Phe^{7}, D-Arg^{8}]-alpha-MSH
[Nle^{4}, D-Phe^{7}, D-Trp^{9}]-alpha-MSH
[Nle^{4}, D-Phe^{7}, D-Lys^{11}]-alpha-MSH
[Nle^{4}, D-Phe^{7}, D-Pro^{12}]-alpha-MSH
[Nle^{4}, D-Phe^{7}, D-Val^{13}]-alpha-MSH
c[Cys^{4}, Cys^{10}]-alpha-MSH
c[Cys^{4}, D-Phe^{7}, Cys^{10}]-alpha-MSH
c[Cys^{4}, Cys^{11}]-alpha-MSH
c[Cys^{5}, Cys^{10}]-alpha-MSH
c[Cys^{5}, Cys^{11}]-alpha-MSH
c[Cys^{4}, Cys^{10}]-alpha-MSH$_{4-13}$
c[Cys^{4}, Cys^{10}]-alpha-MSH$_{4-12}$

[Nle^4, D-Phe^7]-alpha-MSH$_{4-10}$

[Nle^4, D-Phe^7]-alpha-MSH$_{4-11}$

[D-Phe^7]-alpha-MSH$_{5-11}$

[Nle^4, D-Tyr^7]-alpha-MSH$_{4-11}$

[(pNO$_2$)D-Phe^7]-alpha-MSH$_{4-11}$

[Tyr^4, D-Phe^7]-alpha-MSH$_{4-10}$

[Tyr^4, D-Phe^7]-alpha-MSH$_{4-11}$

[Nle^4]-alpha-MSH$_{4-11}$

[Nle^4, (pNO$_2$)D-Phe^7]-alpha-MSH$_{4-11}$

[Nle^4, D-His^6]-alpha-MSH$_{4-11}$

[Nle^4, D-His^6, D-Phe^7]-alpha-MSH$_{4-11}$

[Nle^4, D-Arg^8]-alpha-MSH$_{4-11}$

[Nle^4, D-Trp^9]-alpha-MSH$_{4-11}$

[Nle^4, D-Phe^7, D-Trp^9]alpha-MSH$_{4-11}$

[Nle^4, D-Phe^7]-alpha-MSH$_{4-9}$

[Nle^4, D-Phe^7, D-Trp^9]-alpha-MSH$_{4-9}$

Preferred compounds include:

[Nle^4, D-Phe^7]-alpha-MSH

[Nle^4, D-Phe^7]-alpha-MSH$_{4-10}$

[Nle^4, D-Phe^7]-alpha-MSH$_{4-11}$

[Nle^4, D-Phe^7, D-Trp^9]-alpha-MSH$_{4-11}$

[Nle^4, D-Phe^7]-alpha-MSH$_{4-9}$

The most preferred alpha-MSH analogue for use in the methods of this invention is [Nle^4, , D-Phe^7]-alpha-MSH, referred to hereinafter as "melanotan-1" or "MT-1".

The compounds useful in this invention may be administered by a variety of routes including oral, parenteral or transdermal. The term "parenteral" is used herein to encompass any method by which the compounds according to the present invention are introduced into the systemic circulation and include intravenous, intramuscular and subcutaneous injections. The term "transdermal" as used herein encompasses the administration of the compound by topical methods such as buccal or skin

patches, intranasal or tracheal sprays, by solution for use as ocular drops, by suppositories for vaginal or anal routes of administration or by conventional topical preparations such as creams or gels for localised percutaneous delivery.

The compounds will be formulated in suitable compositions determined by the intended means of administration, according to methods and procedures well-known to those skilled in the art (see, for example, *Remington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing Company, Pennsylvania, USA). For example, the compounds suitable for use in this invention may be formulated or compounded into pharmaceutical compositions comprising at least one compound of the present invention (the compositions may comprise one compound or admixtures of compounds according to the present invention) in admixture with a solid or liquid pharmaceutical excipient such as a diluent or carrier for oral or parenteral administration. As injection medium, water containing the usual pharmaceutical additives for injection solutions, such as stabilising agents, solubilising agents, and buffers is preferred. Among additives of this type are, for example, tartrate and citrate buffers, ethanol, complex forming agents such as ethylenediamine-tetraacetic acid, and high molecular weight polymers such as liquid polyethylene oxide for viscosity regulation. Solid carrier materials include, for example, starch, lactose, mannitol, methyl cellulose, talc, highly dispersed silicic acid, high molecular weight fatty acids such as stearic acid, gelatine, agar-agar, calcium phosphate, magnesium stearate, animal and vegetable fats, and high molecular weight polymers such as polyethylene glycols. Compositions suitable for oral administration can, if desired, contain flavouring and/or sweetening agents. For topical administration, the compounds may be preferably used with various conventional bases for topical preparations such as creams, ointments, gels, lotions or sprays, depending upon the desired mode of delivery of the ingredients to an individual. In manufacturing these preparations, the composition may also be mixed with conventional inert excipients such as thickening agents, emollients, surfactants, pigments, perfumes, preservatives, fillers and emulsifiers, all of which are well known and conventionally used in the formulation of transdermal or other preparations. Typically, these non-active ingredients will make up the greater part of the final preparation. Preferably, the compositions are manufactured to allow for controlled and/or sustained-release delivery.

The actual amount of administered compound according to the present invention may vary between fairly wide ranges depending upon the mode of administration, the excipients used, and the degree of stimulation desired. Such amounts are well within the skill of the pharmaceutical scientist to

determine, and the amount administered to the mammal may be any amount chosen to stimulate melanotropic activity, for example, by formulation as an implant using poly (D, L lactide-co-glycolide polymer[24] or a similar biodegradable, biocompatible polymer as carrier.

In the work leading to the present invention, described in detail in the Example below, two clinical trials of a superpotent melanotropic peptide, melanotan-1 (MT-1), were performed in normal human volunteers with tanning skin types 3-4 (Fitzpatrick scale). The first study in 12 subjects used 0.16 mg/kg/day for 10 days plus UV-B radiation to the buttock to evaluate tanning synergy. The results show significant tanning in the MT-1 treated subjects, and especially at the UV-B-irradiated buttock skin sites. Immunologic parameters were unaltered in 7 of these subjects. A second study randomized subjects to placebo, plus 3-5 days of sunlight to the back, (n =3), or sunlight plus MT-1 at 0.16 mg/kg/day x 20 days over 4 weeks, (n = 5). There was significant whole-body tanning in the MT-1 group and the back areas required 50% less sunlight for equivalent tanning. In addition, tanning of the back area was maintained for over 3 months in the MT-1 treated group compared to 6 weeks in the controls. These results establish that MT-1 synergises with sunlight to produce a dark and long-lasting skin pigmentation.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure I Shows results of a clinical trial and compares mean (n=3) reflectance changes on the back over 6 weeks for the control group (only sunlight) at the sun-exposed back (squares) or the opposite, non-exposed site, (circles). The reflectance values are luminance (solid symbols) and b- scale (blue-yellow) hue (open symbols).

Figure 2 Shows results of a clinical trial and compares mean (n=3) reflectance changes on the back over 10 weeks for subjects receiving sunlight to the back at the start of MT-1 dosing. Tanning is indicated by reduced luminance (squares) and increased b-scale hue (circles) for the sun-exposed back site (solid symbols) and non-exposed back site (open symbols).

Figure 3 Shows results of a clinical trial and compares mean reflectance changes on the back over 10 weeks for subjects (n=2) receiving sunlight to the back starting 1 week after finishing MT-1. Tanning is indicated by reduced luminance (squares) and

increased b-scale hue (circles) for the sun-exposed back site (solid symbols), and the non-exposed back site (open symbols).

The present invention is further described by reference to the following non-limiting Example.

EXAMPLE

Materials and Methods:

Design: Two clinical trials were performed from 1991 to 1994 at the University of Arizona Medical School campus to evaluate the response of human skin to MT-1 when combined with sunlight or simulated ultraviolet-B-range, (UV-B), radiation. Normal subjects with a tanning skin type by history were treated with subcutaneous Melanotan I, (MT-1), at 0.16 mg/kg/day for two to four weeks. The effects on skin pigmentation were evaluated by serial reflectance measurements at 8 anatomic sites, beginning before treatment, at the end of treatment and for up to 4 weeks after MT-1 treatment. Placebo controls were used for Protocol 2 (MT-1 plus mid-day sunlight to half of the back area). Protocol 1 randomized subjects to UV-B exposure on the buttock at either the start of MT-1 treatment (Group A), or immediately after finishing the ten-day course of MT-1 (Group B)

Subjects: Normal subjects were recruited from newspaper ads and were screened to have Type 3-4 skin by the Fitzpatrick scale[3], and for the lack of any history of skin conditions, including skin cancers, dysplastic nevus syndrome or atypical moles. All subjects were required to have normal laboratory values as assessed by serial chemistry (SMAC-20), CBC and urinalysis. Any women must have tested negative for pregnancy and agreed to avoid becoming pregnant by means of active contraception. Additional lab tests that were required by the FDA to be monitored included serum cortisol, LH and FSH, which were measured before treatment and at the end of the two week treatment period.

Melanotan-I: Nle[4]-D-Phe[7] alpha melanocyte-stimulating hormone, (MT-1), was prepared by solid-phase chemistry under GMP conditions at Bachem Inc, Torrance Ca. The white powder was reconstituted in bacteriostatic sodium chloride for injection and tested negative for pyrogens by the Limulus amebocyte lysate (LAL) assay[4]. It was stored frozen prior to thawing immediately prior to subject injections. All doses were subcutaneously administered into the upper arm or thigh using a

25 gauge needle. The doses were calculated using actual body weight to deliver 0.16 mg/kg/day of MT-1 per day. MT-1 was administered daily on Monday to Friday, for two consecutive weeks in Protocol 1 (10 total injections), and for 4 weeks in Protocol 2 (20 total injections). The 0.16 mg/kg dose was used for these Protocols following a Phase I study in a small number of subjects that showed this was the maximally effective daily dose for tanning with minimal side effects[9].

UV Radiation was delivered by means of a solar simulator for Protocol 1, or by a series of timed mid-day sun exposures in the months of March through June, for Protocol 2.

Endpoints for both protocols included pigmentation measured as skin reflectance at eight different anatomic body sites: the forehead, cheek, dorsal neck, inner forearm, scapula, abdomen, buttock, and anterior leg. Skin chromaticity was measured by light reflectance recorded on a Minolta CR200 Chromameter® (Minolta Camera, Osaka, Japan). The reflected light is received and split into three fractions. Luminance (or L-scale), indicates relative brightness from black to white and decreases with tanning. There are two colour scales; the a-scale for yellow to red, which does not change with tanning, and the b-scale which indicates blue-yellow hue and increases with tanning[15-17] . For these studies, only L-scale and b-scale responses were recorded and stored on a portable computer. Eight measurements per anatomic site are performed. Each subject served as his own control and reflectance was serially measured: from baseline (pre-dosing), then at the end of dosing and for several weeks thereafter, usually until the reflectance values returned to the baseline level for each subject. All subjects had their minimal erythema dose, (MED), of UV-B radiation defined at the outset of each study. The MED was defined using a series of graded doses of UV-B delivered from a solar irradiation simulator (Model 600 Multiport® Solar Ultraviolet Simulator, Solar Light Co., Philadelphia, PA).

A secondary biologic endpoint was evaluated in Protocol 1. Seventeen different immunologic parameters were evaluated by flow cytometry tests on blood samples drawn for 7 patients treated with MT-1 This involved the quantification of distinct immune function cell types in peripheral blood, including pan B- cells and several T- lymphocytes subsets: natural killer (NK)T- cells, lymphokine activated killer (LAK) cells, CD-8 (suppressor) and CD-4 (helper) cells, IL-2 receptor + (CD3) cells, transferrin receptor positive T-cells, and three classes of T-cells found in skin (CD4/CD4RP, LFA-3 and HECA452/CD3 T-cells). For these studies, blood was withdrawn by peripheral venipuncture at two pre-dosing times, again at the completion of the ten subcutaneous doses, and then ten days

after completion of dosing, a time at which the tanning response is generally maximal. The white blood cell fraction was separated by Ficol[R] centrifugation and different subtypes of lymphocytes and monocytes were detected and quantified by automated fluorescence-activated cell sorting (Facscan[R], Becton-Dickinson, San Jose, California).

Statistical analyses were performed on the reflectance (tanning) data in both protocols using analysis of variance with a post-test, the Student Neuman Keuls test, for any significant ($p<0.05$) ANOVA differences. To be considered a significantly darkened site required statistical changes in both the L-scale and the b-scale, each at $p < 0.05$ for ANOVA followed by the multiple range test. The cumulative exposure time of sunlight exposure to a given level of skin tanning in Protocol 2 was also compared using ANOVA. The immunologic parameters were compared at two baseline time points and then on the day of dose 5, the day of dose 10 and ten days after dosing ended. The 95% confidence intervals were analysed for the two baseline values, and then between the mean of those baselines and the other time points. The Bonferroni adjustment was used to correct the p-value for the multivariate tests, such that a significant difference would be 0.05/17 (the number of immunologic variables), or $p < 0.0029$ for significance.

Results:

Protocol 1: Effect of Melanotan 0.16 mg/kg/day plus Five Daily Doses of UV-B Radiation at the Beginning or End of MT-1 Dosing Period

The goal of this study was three-fold: (1) determine the effect of tanning at double the dose of MT-1 from the prior trial; (2) evaluate whether there was additive tanning if UV-B radiation was given at the beginning or end of MT-1 dosing period; and, (3) evaluate immune function parameters in subjects given MT-1. There were 12 subjects with Fitzpatrick skin types 3-4 randomized to this study. Group A, ($n = 7$), received UV-B radiation to the buttock on the first five days of MT-1 dosing. Group B, ($n = 5$), received the same three dose levels of UV-B radiation to the buttock for five consecutive days, starting 3 days after the last dose of MT-1 was administered. An individual subject's MED was determined visually following graded UV-B exposure to the opposite buttock prior to MT-1 dosing. The MED was defined as the amount of radiation, from 15.75 to 42 mjoules/cm^2, that produced faint erythema with four distinct borders, measured 24 hours after UV-B dosing. When combined with MT-1, the UV-B radiation was delivered daily for five consecutive days to the buttock area at three solar

simulator settings of 0.25, 0.50 and 0.75, representing 5.25 mjoules/cm^2, 10.5 mj/cm^2 and 15.75 mjoules/cm^2, respectively. The MT-1 doses for both groups were administered by subcutaneous injection into the upper arm on Monday-Friday, for two consecutive weeks. There was a total of ten doses, each delivering 0.16 mg/kg. Characteristics of each group are summarised in Table 1.

Table 1: Skin Type Characteristics for Subjects Receiving MT-1 0.16 mg/kg Plus Five Days of UV-B Radiation to the Buttock

Subject Number	Sex	Age	Skin Type[1]	MED (mjoules)	Days of UV-B[2]
063	F	54	IV	31.5	1-5
067	M	24	III	21.0	1-5
069	F	52	IV	26.25	1-5
070	F	45	III	26.25	1-5
072	F	36	III	36.75	1-5
073	F	51	IV	31.5	1-5
078	M	29	III	26.25	1-5
065	M	41	IV	31.5	15-19
066	M	27	III	26.25	15-19
071	F	49	III	31.5	15-19
074	M	24	IV	26.25	15-19
077	F	40	IV	26.25	15-19

[1]Fitzpatrick scale for tanning vs. burning by personal history
[2]Five daily doses of UV-B to the buttock at 0.25, 0.5 and 0.75 of MED radiation

Tanning Results for the eleven subjects are summarised in Table 2. There was significant skin darkening at some body site in all but one individual, indicating an overall response rate of 10/11 subjects. The non-responsive subject was No. 073 (see Table 1 for characteristics). The sites of significant skin pigmentation varied for different subjects, and the most responsive skin sites were the forehead, cheek and scapula with 6/11 subjects responding at each site. Curiously, the neck was much less responsive with only 3/11 subjects exhibiting significant changes in both luminance and b-scale reflectance values from baseline. The results in Table 2 differ from our previous studies in that non-responsive sites in the past, such as the buttock, abdomen and anterior leg, exhibited significant skin darkening in 4 or 5 of the total 11 subjects in this trial. Another difference is the prolonged duration of significant darkening. The results in Table 2 also show that most subjects had not returned to their baseline reflectance values at the final evaluation at week 6 (4 weeks after MT-1 dosing ended). Finally, there was significant enhancement for MT-1 and the five UV-B doses delivered to the buttock on both the early "A" schedule, or the later "B" schedule.

Table 2 Reflectance Changes in 11 Subjects Given Melanotan-10.16mg/kg/day for 10 Injections

		Mean Change in Absolute Luminance (No. Subjects Significantly Different)[2];			
	Mean Reflectance at Baseline	Week 2	Week 3	Week 4	Week 6
Anatomic Site	Luminance[1]				
Forehead	(61.59)	-2.47(5)	-2.85(5)	-2.21(6)	-1.72(4)
Cheek	(61.06)	-2.58(5)	-2.11(5)	-1.92(6)	-1.88(4)
Neck	(60.35)	-2.16(3)	-2.46(3)	-1.98(3)	-2.35(3)
Abdomen	(67.29)	-1.26(2)	-1.75(5)	-1.49(3)	-0.79(3)
Scapula	(64.30)	-0.9(6)	-0.43(4)	-0.67(4)	-0.97(4)
Buttock	(68.25)	-0.24(0)	-1.05(5)	-0.67(1)	-0.46(0)
Forearm	(65.59)	-1.68(4)	-1.19(5)	-1.32(5)	-1.22(3)
Leg Anterior	(64.67)	+0.55(0)	-0.34(4)	0.04(1)	-0.32(3)

[1]Mean baseline luminance for all 11 subjects.
[2]Number of subjects (in parentheses) of total 11 showing a significant decrease in luminance and increase in b-scale (data not shown for b- scale differences).

Side effects in Protocol 1 were quantitatively and qualitatively similar to the previous studies[7,9,13]. The most common side effect was flushing of the upper body that occurred variably within minutes after MT-1 injection. About half of the subjects experienced a median of three instances of this self-limited reaction at some time during the two week dosing period. These reactions lasted from a few minutes up to an hour and were not associated with other sequelae. A mild sensation of nausea was reported in 4 of the 11 subjects. This effect was typically noted after the second or third injection of MT-1 and lasted for 30 minutes up to several hours. Because of the mild severity, antiemetic therapy was not required in any subject, but a few subjects described mild anorexia late in the evening on injection days. Fatigue was also reported in about one-third of the subjects, usually in the afternoon of the day of injection. This was variable in intensity, but was never severe enough to require bed rest. And, like the flushing reactions, the episodes of afternoon fatigue did not recur or increase in intensity with successive doses.

Immunologic Findings: Seven subjects in this protocol had five blood samples collected before, during and after receiving MT-1 to determine whether the acute drug regimen induced changes in 17 different types of white blood cells. The first two samples were baselines, drawn prior to dosing, about 8 weeks apart and were compared statistically. This analysis showed that for all 17

parameters in the two baselines, zero was contained in the 95% confidence interval. The average of these two baselines was then calculated and used for comparison to the other three time points: on the day of doses 5 and 10, and ten days thereafter. The results show that two parameters, T-memory cells ($p = 0.05$), and T-cell LCA2 cells ($p = 0.01$), were approximately doubled at the time of dose 5. At the time of dose 10, the T-helper LCA cell levels were decreased by about 50%, ($p = 0.01$). There were no significant changes noted at the last time point, ten days after the last dose was delivered. However, these individual statistical differences did not remain significant after applying the Bonferroni correction for multiple analyses.

Protocol 2: Effect of Prolonged Melanotan-1 Combined with Sunlight to the Back

This open-label trial in 8 subjects with type 3-4 skin evaluated the effects of a prolonged schedule of MT-1 at 0.16 mg/kg/day for twenty injections (Mon-Fri/week) over 4 weeks. This was combined with full sunlight exposure to one-half of the back for 3-4 days, until a visual tan was apparent. The sunlight exposures were randomized to be given either at the start of MT-1 dosing (n=3), or after ten of the total 20 doses had been administered (n=2). One student subject in the latter group dropped out mid-way through dosing because of time commitments and therefore only two subjects are available for analysis. A control group of three subjects received the same sun exposure regimen to the back without any MT-1 to allow for a comparison of the time to achieve comparable tanning of the exposed hemi-back site.

Table 3 summarises the sun-response characteristics of the subjects in this trial. The mean (SD) sun exposure time required for a visually perceptible tan on the exposed back site in the MT-1 group was 87 (4.5) minutes. This was delivered over a median of three days, with each daily exposure averaging 30 minutes. By comparison, in the sunlight-only control group, a median of five exposures of 25-35 minutes each were required to achieve the same degree of tanning at the exposed back site. The total mean (SD) sun exposure time in this group was 165 (15) minutes, double that in the MT-1 group ($p < 0.001$).

Table 3: Characteristics of Subjects Receiving Sunlight With or Without a Prolonged Course Melanotan-1

No.	Age	Sex	Skin Type	MED (mjoules)	Sun Exposure for a Visually-Apparent Tan: Days (Minutes)	Total (Min)	MT-1 (days)
813	34	M	3	15.75	1(30), 2(20), 3(40)	90	1-20
814	24	M	3	21.0	2(20), 3(30), 5(40)	90	1-20
815	25	M	4	26.25	1(15), 2(15), 3(20) 18(20), 10(20)	90	1-20
816	22	M	3	15.75	13(25), 14(30), 15(25)	80	1-20
817	39	M	3	21.0	13(30), 14(25), 15(30)	85	1-20
821	44	M	3	21.0	1(35), 2(35), 3(30), 4(30), 5(35)	165	None
820	32	M	3	15.75	1(35), 2(30), 3(25) 4(35), 5(30), 6(25)	180	None
821	23	M	4	26.25	1(30), 2(35), 3(30) 4(30), 5(25)	150	None

The three control subjects developed significant darkening of the sun-exposed back that involved a mean 7.5 unit decrease in luminance and a 4 unit increase in mean b-values (Figure 1). As expected; these tans were limited to the sun-exposed site, and the non-exposed back sites actually lightened over the course of the study (Figure 1, circle symbols,). In addition, the sun-exposed back sites had returned to baseline reflectance values within 5 weeks of sun exposure. Figure 2 compares the effect of combination sunlight exposure, begun on the first day of MT-1 dosing, with reflectance performed on the subjects adjacent non-sun-exposed back site. This comparison shows that the combination produced rapid and profound skin darkening at the sun-exposed back site (Figure 2, solid symbols). This was significantly greater than the darkening produced by MT-1 alone at the adjacent (unexposed) back site (Figure 2, open symbols). The absolute change in reflectance units for the combination was profound involving the largest reflectance changes we have ever recorded: a 10-15 unit decrease in luminance, and a 5-10 unit increase in b-values.

When sun-exposure was added to MT-1 at the end of the first two weeks of dosing, there was a similar increase in darkening at the sun-exposed back site, compared to an adjacent non-sun exposed back site (Figure 3). However, in this case, the onset of darkening was delayed by 1-2 weeks. This represents a significant difference when compared to sunlight on the first day of MT-1 dosing. Figures 2 and 3 show that the duration of darkening was significantly prolonged for all subjects receiving sunlight-plus MT-1. In this case, it did not matter whether sunlight was added at

the start or middle of the MT- 1 dosing period. Indeed, at the conclusion of reflectance monitoring at 11 weeks, all MT-1 treated subjects still maintained significant tanning of the both sun-exposed and non-exposed back sites. This differs dramatically from the sun-only controls, wherein reflectance values had all returned to baseline after 5 weeks (Figure 1). The difference is even more remarkable when one considers that the controls received almost twice as much total sun exposure to the back.

Since the group receiving MT-1 in Protocol 2 experienced the greatest cumulative drug exposure to date, the question of side effects has special importance. The most common side effect was facial and upper truncal flushing which occurred variably. There were nine instances of flushing in 3 of the 5 subjects. As in our prior studies, the onset was within minutes of MT-1 injection and it typically resolved with 30-60 minutes. The most serious side effect was nausea, which was experienced by two of the five subjects. This began within 40 minutes of the first injection number in subject 817. To prevent nausea, the next three injections in this subject were given after 10 mg of prochlorperazine (Compazine^{R}), had been administered orally. Another subject, No. 816, also experienced nausea after the second dose and received 10 mg of oral prochlorperazine before the next three doses. Later MT-1 doses were given with no antiemetics and there was no significant nausea. The only other reported side effect was afternoon fatigue or somnolence, which was reported in 3 subjects. For example, during week three, one subject described a two hour period of fatigue after each injection. In the other subject, general fatigue was described throughout the second week of the injections with some persistence over the weekend when no MT-1 was administered. None of these side effects were of moderate or severe intensity and there was no evidence of cumulative toxicity. Indeed, most side effects were reported during the first two weeks of the 4 week regimen, and only one instance each of flushing and fatigue were reported in the last (fourth) week of MT-1 dosing.

Discussion:

The primary goal of the current studies was to characterise the effect of MT-1 combined with UV light. The results show that the synthetic superpotent melanotropin, MT-1, can be safely combined with small amounts of UV-B from a solar simulator, or with brief exposures to full sunlight. The latter combination produced a marked enhancement of skin tanning, with the most rapid onset seen for sunlight added at the start of MT-1 dosing. We have further shown that MT-1 can be administered for 4 weeks at a daily dose of 0.16 mg/kg, without producing cumulative, more intense or different side effects. The 0.16mg/Kg dose of MT-1 is superior to the 0.08 mg/kg dose used in the original clinical

study[7] in terms of both the degree of tanning, as well as the number of anatomic sites which responded by darkening. For example, in our original study of 0.08 mg/kg in 28 male subjects, significant skin darkening was only observed on the forehead, cheek and neck[7]. In contrast, the results from Protocol 1, show that significant darkening can be achieved at most body sites, including in some cases, the buttocks, wherein melanocortin receptor densities are very low[18]. We were also able to demonstrate a significant enhancement at sites receiving concomitant UV-B radiation from the solar simulator. There was one female subject with Type 4 skin by history, who did not respond at any skin site to the 0.16 mg/kg dose of MT-1. This is the first observation of a completely non-responsive individual, and there is no clear explanation at this time.

The native hormone, alpha-MSH, has been reported to have broad anti-inflammatory activities in experimental models of inflammations[10]. These effects include inhibition of arthritis in a rat model[11], reduction of endotoxin-induced liver inflammation in a septic shock model[12]. and improved survival in a model of endotoxemia and peritonitis[10]. These effects may be mediated by alpha-MSH-induced inhibition of the synthesis and activity of cytokines and chemo-attractive chemokines in neutrophils[10]. Alternatively direct effects on neutrophil migration and superoxide dismutase production[9] have been reported. Protocol 1 indirectly addressed the issue of immunologic activity for MT-1 in humans receiving ten injections of 0.16 mg/kg. At the end of the two week dosing period, we could not demonstrate any significant changes in the absolute numbers of 17 different white blood cell subtypes in the peripheral blood of 7 of these subjects. However, the effectiveness of these peripheral blood cells to mount an immunologic reaction was not evaluated, and therefore, we cannot rule out an alteration in immune response induced by MT-1. On the other hand, no infections have been observed in any of the approximately 100 normal subjects treated with MT-1 to date[7,9,13]. Thus, while the question of whether MT-1 induces immunologic alterations in humans is still largely unanswered, we do know that it does not acutely alter the number of several classes of immunologic white blood cells in the peripheral blood. The lack of an immunologic effect for MT-1 is also consistent with a dermal study in mice wherein the native hormone blocked contact hypersensitivity responsiveness, but MT-1 did not[20].

The other side effects of Melanotan-1 seen in this study are similar to those previously reported[7,9,13]. Nausea induced by MT-1 was seen in about 20% of the current subjects and required antiemetic treatment in only two subjects. This effect may be mediated by interaction of MT-1 with melanocortin-3 receptors, (MC3R), which have been found in the gut tissues of animals[21]. The fact

that these mild gastrointestinal side effects were infrequent and were not cumulative, suggests that any activation of MC3R by MT-1 is not dose-limiting. The biochemical pathway for the other MT-1 side effects, notably facial flushing and fatigue, are not known. It is unlikely that the acute flushing reactions in the upper trunk are mediated by melanotropic activation of MC1R. The onset of the reactions are rapid, and more time would be required for the synthesis and release of melanin following MT-1 stimulation. Thus, other vasoactive pathways must be involved in mediating this unusual side effect. The only other side effect, fatigue, was also seen in our prior Phase I dose-escalation study, and was dose-dependent in severity[13]. In our current study, fatigue of a mild nature was noted at some time in about one-third of the subjects. Like the other toxicities, it did not recur with each dose and was not cumulative in intensity when it did recur. Whether these effects are mediated by binding to MC3R and MC4R, found in the brain[22], is not known. This is unlikely however, since a prior (unpublished) pharmacokinetic study could not detect significant drug uptake into the brain in rats given radiolabelled MT-1.

Perhaps the most important observation in the two clinical studies of MT-1 and light, is the observation of marked tanning synergy with the combination of UV-B-light (Protocol 1) and sunlight (Protocol 2). The degree of skin darkening measured at both light exposed sites was significantly greater than that achieved with either light, or drug alone. Indeed, the tanning observed in the sun-exposed back in Protocol 2 is the most intense we have ever measured. Furthermore, the combination of MT-1 plus sunlight produced a long lasting tan at the sun-exposed back sites. This had still not returned to baseline reflectance values 11 weeks after MT-1 dosing started, significantly longer than we have seen previously using a two week course of MT-1 at the 0.16mg/kg dose[13]. The 4 week course of MT-1 used in Protocol 2 also represents the largest cumulative exposure to drug to date. Importantly, we saw no new side effects or more intense side effects with this doubled exposure to MT-1.

Melanotan-1 has high binding affinity for melanocortin 1 receptors, (MC1R) in the epidermis[1]. Possibly due to its high potency in experimental systems, it can activate receptors that have mutations at various sites in the seven transmembrane domains of the molecule, a feature not shared with natural alpha-MSH[23]. This may have important implications for individuals with MC1R gene mutations since these persons tan poorly and are at a higher risk for both basal and squamous cell carcinomas. In addition, other studies suggest that the risk of melanoma is also increased in individuals with MC1R variant alleles[6]. Thus, the availability of a more potent agonist for the MC1R,

raises the possibility that MT-1 could induce a protective tan even in individuals with mutated receptor genes. While this might reduce the risk for all types of sun-induced skin cancers, the current studies did not test this hypothesis . Rather, the subjects in the three current protocols were selected for the ability to respond to sunlight by tanning without burning (Fitzpatrick skin types[3-4]). Therefore, we do not know whether humans with MC1R gene mutations will respond to MT-1.

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CLAIMS:

1. A method for the stimulation of integumental melanocytes in a mammal, which comprises the steps of:

(i) administering to said mammal an amount of alpha-MSH or an alpha-MSH analogue effective to stimulate melanocytes in the skin or other epidermal tissue; and

(ii) exposing said skin or other epidermal tissue to ultraviolet (UV) irradiation.

2. A method for stimulating melanin production in a mammal, which comprises the steps of:

(i) administering to said mammal an amount of alpha-MSH or an alpha-MSH analogue effective to stimulate melanin production in the skin or other epidermal tissue; and

(ii) exposing said skin or other epidermal tissue to ultraviolet (UV) irradiation.

3. A method for inducing tanning in a mammal, which comprises the steps of:

(i) administering to said mammal an amount of alpha-MSH or an alpha-MSH analogue effective to induce tanning in the skin or other epidermal tissue; and

(ii) exposing said skin or other epidermal tissue to ultraviolet (UV) irradiation.

4. The method according to claim 1, claim 2 or claim 3 wherein said mammal is a human.

5. A method for inducing skin tanning in a human which comprises the steps of:

(i) administering to said human an amount of alpha-MSH or an alpha-MSH analogue effective to induce tanning in the skin or other epidermal tissue; and

(ii) exposing said skin or other epidermal tissue to ultraviolet (UV) irradiation.

6. The method according to claim 1, claim 2, claim 3 or claim 5, wherein the alpha-MSH analogue is a compound of the formula:

R_1-W-X-Y-Z-R_2

wherein

R_1 is selected from the group consisting of Ac-Gly-, Ac-Met-Glu-, Ac-Nle-Glu-, and Ac-Tyr-Glu-;

W is selected from the group consisting of –His- and –D-His-;

X is selected from the group consisting of –Phe-, -D-Phe-, -Tyr-, -D-Tyr-, -(pNO_2)D-Phe^7-;

Y is selected from the group consisting of –Arg- and –D-Arg-;

Z is selected from the group consisting of –Trp- and –D-Trp-; and

R_2 is selected from the group consisting of $–NH_2$; -Gly-NH_2; and –Gly-Lys-NH_2.

7. The method according to claim 1, claim 2, claim 3 or claim 5, wherein the alpha-MSH analogue is a compound selected from the group consisting of:

[D-Phe^7]-alpha-MSH

[Nle^4, D-Phe^7]-alpha-MSH

[D-Ser^1, D-Phe^7]-alpha-MSH

[D-Tyr^2, D-Phe^7]-alpha-MSH

[D-Ser^3, D-Phe^7]-alpha-MSH

[D-Met^4, D-Phe^7]-alpha-MSH

[D-Glu^5, D-Phe^7]-alpha-MSH

[D-His^6, D-Phe^7]-alpha-MSH

[D-Phe^7, D-Arg^8]-alpha-MSH

[D-Phe^7, D-Trp^9]-alpha-MSH

[D-Phe^7, D-Lys^{11}]-alpha-MSH

[D-Phe-^{7}, D-Pro^{12}]-alpha-MSH

[D-Phe^7, D-Val^{13}]-alpha-MSH

[D-Ser^1, Nle^4, D-Phe^7]-alpha-MSH

[D-Tyr^2, Nle^4, D-Phe^7]-alpha-MSH

[D-Ser^3, Nle^4, D-Phe^7]-alpha-MSH

[Nle^4, D-Glu^5,D-Phe^7]-alpha-MSH

[Nle^4, D-His^6, D-Phe^7]-alpha-MSH

[Nle^4, D-Phe^7, D-Arg^8]-alpha-MSH

[Nle^4, D-Phe^7, D-Trp^9]-alpha-MSH

[Nle^4, D-Phe^7, D-Lys^{11}]-alpha-MSH

[Nle^4, D-Phe^7, D-Pro^{12}]-alpha-MSH

[Nle^4, D-Phe^7, D-Val^{13}]-alpha-MSH

c[Cys^4, Cys^{10}]-alpha-MSH

c[Cys^4, D-Phe^7, Cys^{10}]-alpha-MSH

c[Cys^4, Cys^{11}]-alpha-MSH

c[Cys^5, Cys^{10}]-alpha-MSH

c[Cys^5, Cys^{11}]-alpha-MSH

c[Cys^4, Cys^{10}]-alpha-MSH$_{4-13}$
c[Cys^4, Cys^{10}]-alpha-MSH$_{4-12}$
[Nle^4, D-Phe^7]-alpha-MSH$_{4-10}$
[Nle^4, D-Phe^7]-alpha-MSH$_{4-11}$
[D-Phe^7]-alpha-MSH$_{5-11}$
[Nle^4, D-Tyr^7]-alpha-MSH$_{4-11}$
[(pNO$_2$)D-Phe^7]-alpha-MSH$_{4-11}$
[Tyr^4, D-Phe^7]-alpha-MSH$_{4-10}$
[Tyr^4, D-Phe^7]-alpha-MSH$_{4-11}$
[Nle^4]-alpha-MSH$_{4-11}$
[Nle^4, (pNO$_2$)D-Phe^7]-alpha-MSH$_{4-11}$
[Nle^4, D-His^6]-alpha-MSH$_{4-11}$
[Nle^4, D-His^6, D-Phe^7]-alpha-MSH$_{4-11}$
[Nle^4, D-Arg^8]-alpha-MSH$_{4-11}$
[Nle^4, D-Trp^9]-alpha-MSH$_{4-11}$
[Nle^4, D-Phe^7, D-Trp^9]alpha-MSH$_{4-11}$
[Nle^4, D-Phe^7]-alpha-MSH$_{4-9}$
[Nle^4, D-Phe^7, D-Trp^9]-alpha-MSH$_{4-9}$.

8. The method according to claim 7, wherein the alpha-MSH analogue is a compound selected from the group consisting of:
[Nle^4, D-Phe^7]-alpha-MSH
[Nle^4, D-Phe^7]-alpha-MSH$_{4-10}$
[Nle^4, D-Phe^7]-alpha-MSH$_{4-11}$
[Nle^4, D-Phe^7, D-Trp^9]-alpha-MSH$_{4-11}$
[Nle^4, D-Phe^7]-alpha-MSH$_{4-9}$

9. The method according to claim 8, wherein the alpha-MSH analogue is [Nle^4,D-Phe^7]-alpha-MSH.

10. The method according to claim 1, claim 2, claim 3 or claim 5, wherein said step of exposing to UV irradiation is carried out subsequent to said step of administration of alpha-MSH or an alpha-MSH analogue.

11. The method according to claim 1, claim 2, claim 3, or claim 5, wherein said administration is oral, parenteral or transdermal administration.

12. The method according to claim 1, claim 2, claim 3 or claim 5, wherein said ultraviolet (UV) irradiation consists of or comprises UV-B irradiation.

13. Use of alpha-MSH or an alpha-MSH analogue in a method for the stimulation of integumental melanocytes in a mammal, which comprises the steps of:

(i) administering to said mammal an amount of said alpha-MSH or alpha-MSH analogue effective to stimulate melanocytes in the skin or other epidermal tissue; and

(ii) exposing said skin or other epidermal tissue to ultraviolet (UV) irradiation.

14. Use of alpha-MSH or an alpha-MSH analogue in a method for stimulating melanin production in a mammal, which comprises the steps of:

(i) administering to said mammal an amount of said alpha-MSH or alpha-MSH analogue effective to stimulate melanin production in the skin or other epidermal tissue; and

(ii) exposing said skin or other epidermal tissue to ultraviolet (UV) irradiation.

15. Use of alpha-MSH or an alpha-MSH analogue in a method for inducing tanning in a mammal, which comprises the steps of:

(i) administering to said mammal an amount of said alpha-MSH or alpha-MSH analogue effective to induce tanning in the skin or other epidermal tissue; and

(ii) exposing said skin or other epidermal tissue to ultraviolet (UV) irradiation.

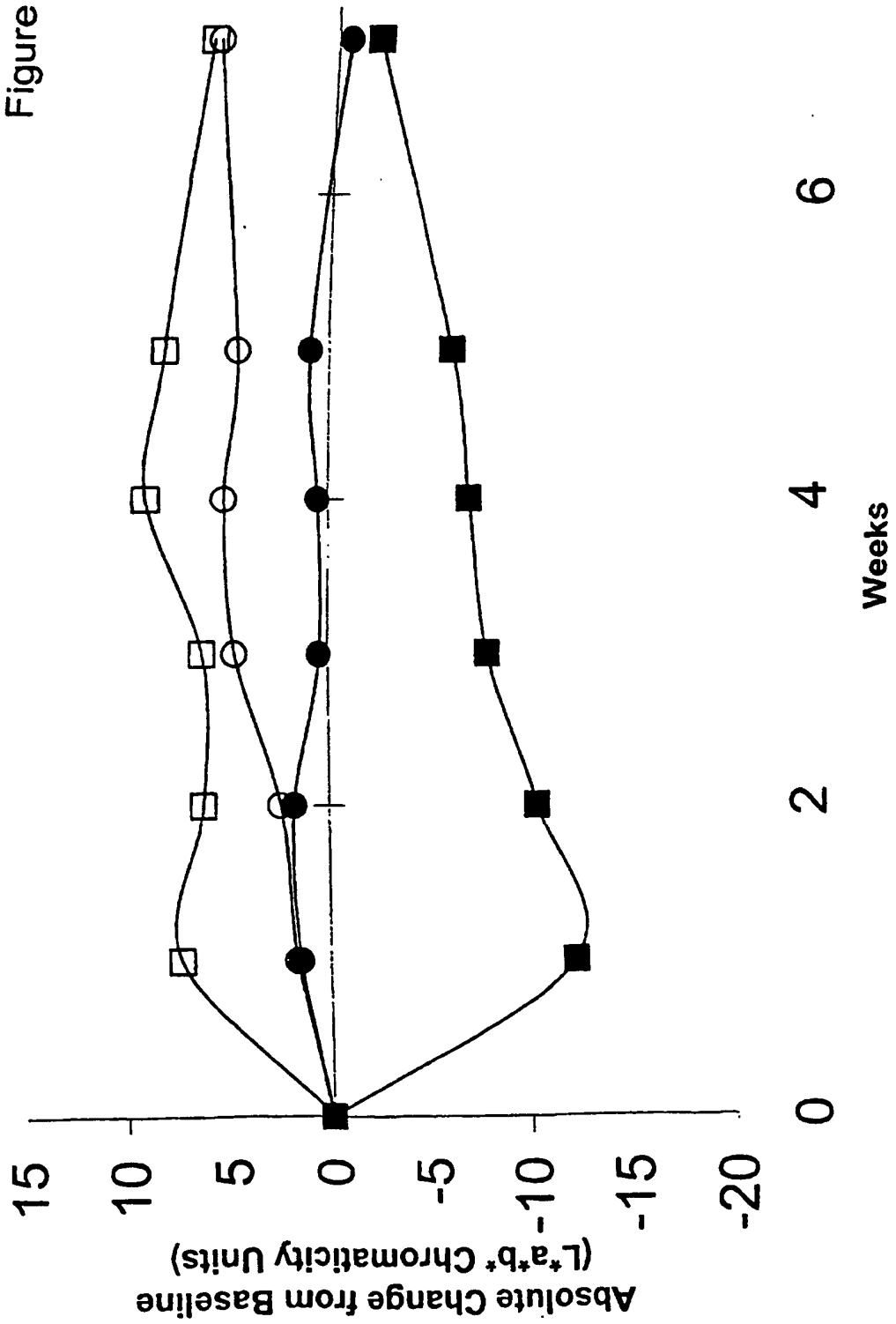
16. The use according to claim 1, claim 2 or claim 3 wherein said mammal is a human.

17. Use of alpha-MSH or an alpha-MSH analogue in a method for inducing skin tanning in a human which comprises the steps of:

(i) administering to said human an amount of said alpha-MSH or alpha-MSH analogue effective to induce tanning in the skin or other epidermal tissue; and

(ii) exposing said skin or other epidermal tissue to ultraviolet (UV) irradiation.

Figure 1


Absolute Change from Baseline
(L*a*b* Chromaticity Units)
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-20
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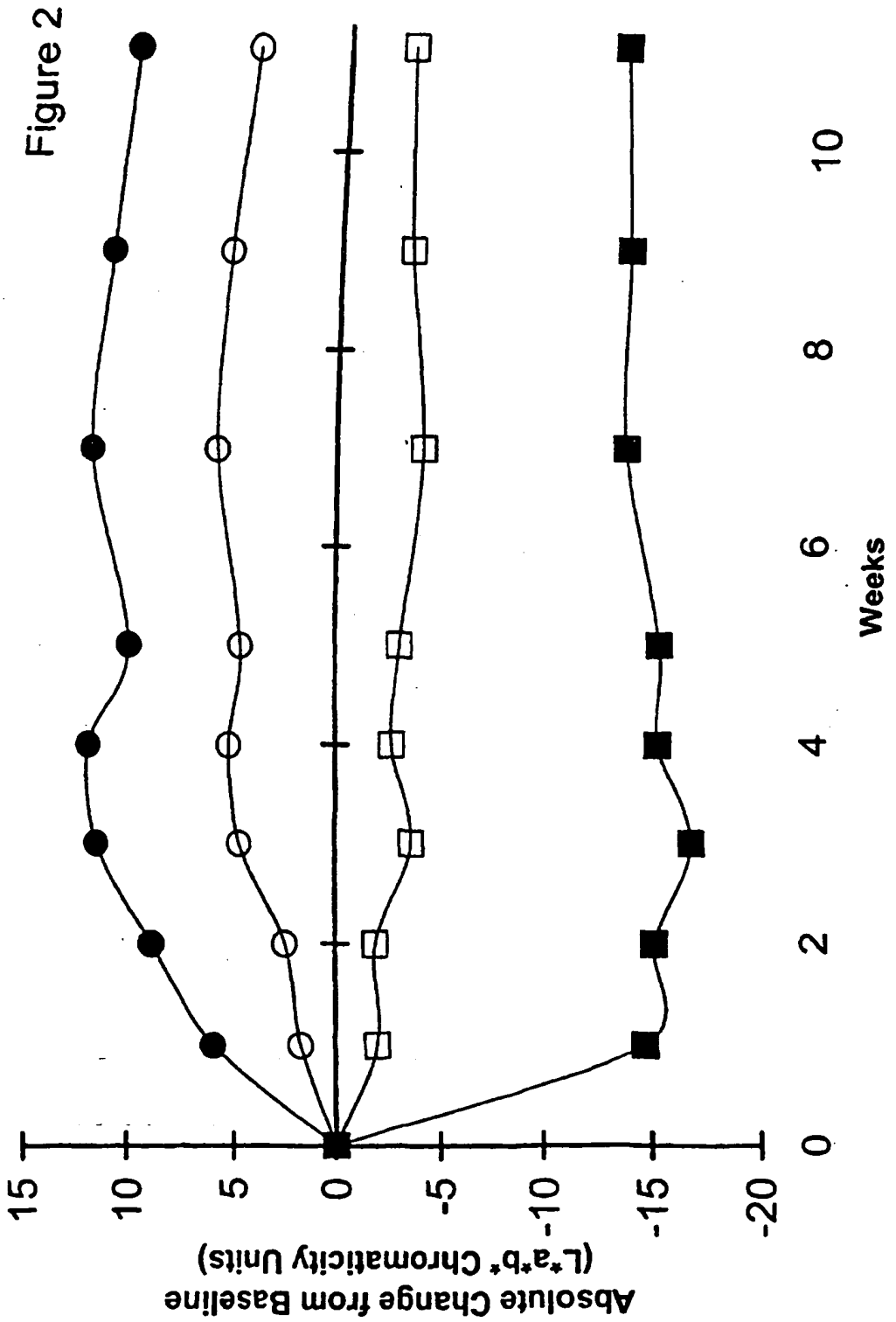
2
4
6
Weeks

Figure 2


Absolute Change from Baseline
(L*a*b* Chromaticity Units)
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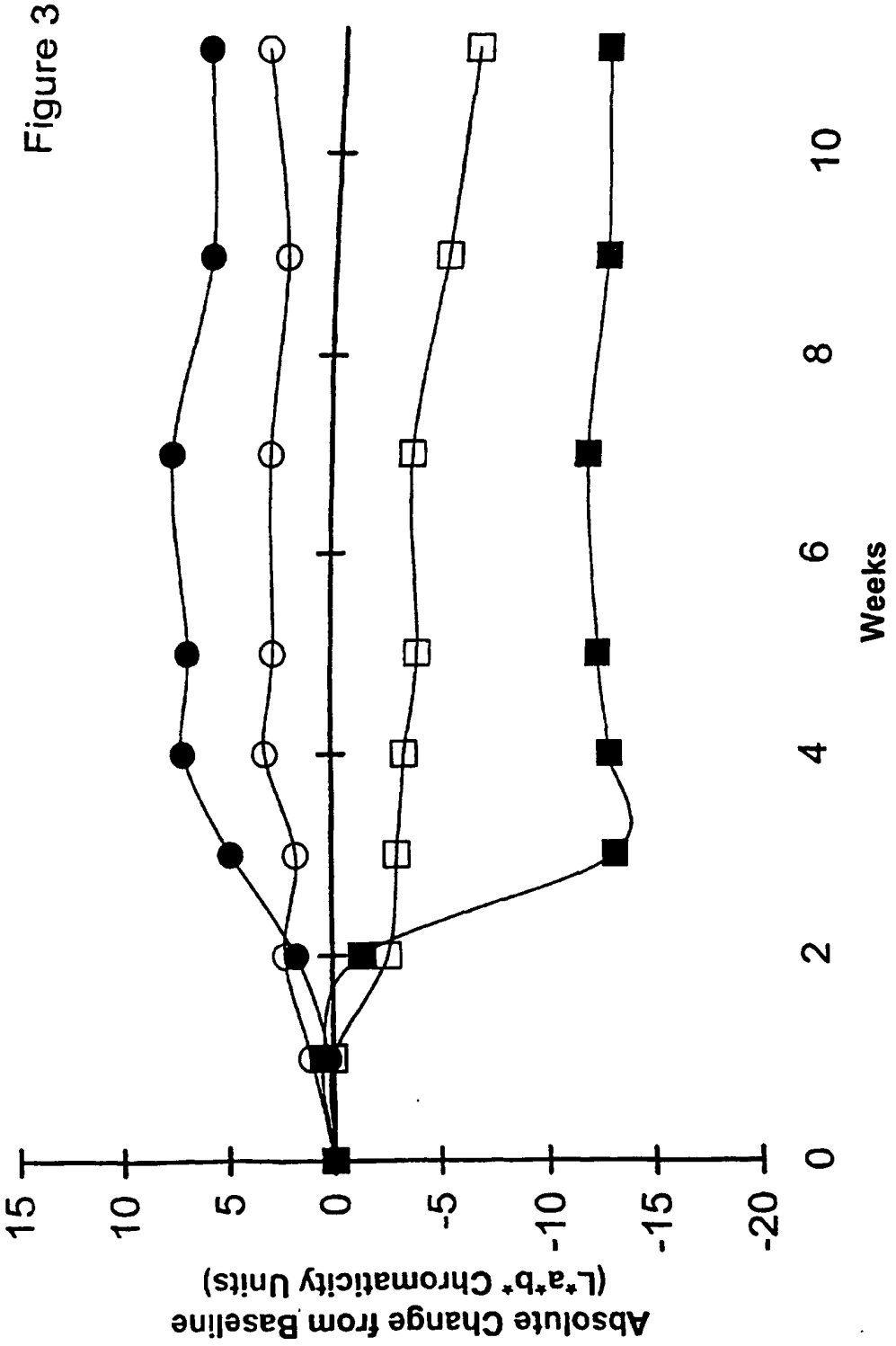
-10
-15
-20
0
2
4
6
8
10
Weeks

Figure 3


Absolute Change from Baseline
(L*a*b* Chromaticity Units)
15
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-5
-10
-15
-20
0
2
4
6
8
10
Weeks

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU03/00230

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: A61K 7/021, 7/42, 38/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DWPI, MEDLINE; Keywords; MSH, Melanocyte Stimulating Hormone, Melanotan-1, MT-1, UV, Ultraviolet, Sun, Sunlight, Tan, Tanning

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Li W, Hill HZ, Induced melanin reduces mutations and cell killing in mouse melanoma, Photochem Photobiol, Mar 1997, 65(3) pages 480-5. abstract, page 481 left column paragraph 3.	1-2, 4, 10-12, 13-14, 16
X	WO 87/04623 A (University Patents Inc) 13 August 1987. entire document.	1-17
X	Virador VM et al, Influence of alpha-melanocyte-stimulating hormone and ultraviolet radiation on the transfer of melanosomes to keratinocytes, FASEB, January 2002, 16(1), pages 105-7. abstract	1-17

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 March 2003	07 APR 2003
Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	TERRY SUMMERS Telephone No : (02) 6283 3126

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU03/00230

C (Continuation). **DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Levine N et al, Induction of skin tanning by subcutaneous administration of a potent synthesis melanotropin, JAMA, 20 Nov 1991, 266(19) pages 2730-2736 abstract	1-17
P, X	Epitan: Announcements: "Testing of Anti-sunburn Drug Underway", 30 January 2003, (retrieved 13/03/2003) Retrieved from internet URL: http://www.epitan.com.au/news_announcements.aspx?view=10 Entire document	1-17

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU03/00230

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	8704623	AU	70828/87	CA	1282324	DK	5181/87
		EP	259440	NZ	233248	US	4866038
		US	4918055				

END OF ANNEX

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
29 December 2004 (29.12.2004)

PCT

(10) International Publication Number
WO 2004/113389 A2

(51) International Patent Classification[7]: C07K 17/00

(21) International Application Number:
PCT/DK2004/000461

(22) International Filing Date: 28 June 2004 (28.06.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

PA 2003 00963	26 June 2003 (26.06.2003)	DK
60/482,452	26 June 2003 (26.06.2003)	US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for all designations*

— *of inventorship (Rule 4.17(iv)) for US only*

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: HIGH CAPACITY POLY(ALKYLENE)GLYCOL BASED AMINO POLYMERS

(57) Abstract: The present invention relates to a cross-linked and beaded, stable and high loading capacity polymer matrix for affinity chromatography applications and for solid phase synthesis. The polymer matrix can be obtained by a method comprising the steps of providing a plurality of macromonomers each comprising a poly(oxalkylene) chain terminated with an acylamide functional group, polymerising said macromonomers using a free radical initiator or an ionic initiator, optionally with the addition of copolymerizing agents, and converting in the beaded polymer matrix at least 50% of the amide groups to amine functional groups by reduction of the amide groups with a suitable reducing agent.

WO 2004/113389 A2

High capacity poly(alkylene)glycol based amino polymers

This application is a non-provisional of U.S. provisional application Serial No. 60/482,452 filed on 26 June 2004, which is hereby incorporated by reference in its entirety. All patent and non-patent references cited in the application, or in the present application, are also hereby incorporated by reference in their entirety.

Field of invention

The present invention relates to the development of a beaded, stable and high loading capacity resin for affinity chromatography applications and for solid phase synthesis. The exhaustive reduction of amide groups in highly crosslinked amide based polymers comprising poly(oxyalkylene) chains terminated with acrylamide functional groups copolymerized with other modifying agents form a stable polymer resin with a high amine functional loading.

The polymer comprises a crosslinked poly(alkylene)glycol network which has a unique, three dimensional structure and can be applicable e.g. as a chromatographic resin or as a solid support for the synthesis of peptides, oligonucleotides, oligosaccharides, or as a substrate for the immobilization of proteins.

Background of invention

Wide ranges of conventional homogeneous solution phase organic reactions are now successfully performed on solid supports. However this has required suitable polymeric materials. The development and exploitation of solid-phase combinatorial chemistry technology has rapidly evolved based on the pioneering work of Merrifield [Merrifield, J. Am. Chem. Soc., 85, 2149 (1963)].

Merrifield introduced the divinyl benzene crosslinked polystyrene for solid phase synthesis and it has been widely used until now with some refinements. The initial revelations of its use focused on the solid-phase synthesis of oligomers of amino acids or nucleotides, or on unnatural oligomers of other chemical building blocks like peptoids [Geysen et al., J. Bioorg. Med. Chem. Lett., 3, 397 (1993); Egholm et al., J. Am. Chem. Soc., 114, 1895 (1992); Simon et al., Proc. Natl. Acad. Sci. USA, 89,

9367 (1992)]. Recently, the library synthesis of nonoligomeric small molecules has become an area of intense research activity [Wang et al., J. Med. Chem., 38, 2995 (1995)].

In any approach to produce chemical library, whether it is solid-phase or solution-phase, is the need of rapid purification, isolation, and manipulation of chemical library members during their intermediate and final synthetic steps of preparation. The solid-phase technology offers advantages like ease of separating the products from the reaction medium and the manipulation of the beads using volumetric techniques. Due to the high rigidity and hydrophobicity the PS-DVB resin it is not suitable for all conventional organic synthesis and is often not suitable for the subsequent screening of the libraries on solid support.

In order to overcome the above-mentioned difficulties, a series of resins such as poly(ethyleneglycol) polystyrene (PS-PEG) [Zalipsky et al., React. Polym., 22, 243 (1994)], tentagel graft resin [Hellerman et al., Makromol Chem., 184, 2603 (1983)], alkanediol diacrylate crosslinked polystyrene [Renil et al., Tetrahedron, 50, 6681 (1994); Varkey et al., J. Peptide Res., 51, 49 (1998); Roice et al., Macromolecules, 32, 8807 (1999)], polyamides [Arshady et al., J. Chem. Soc., Perkin Trans. 1, 529 (1981)], cotton and other carbohydrates [Englebresten et al., Int. J. Peptide Protein Res., 48, 546 (1994)], PEGA [Meldal, Tetrahedron Lett., 33, 3077 (1992)], CLEAR [Kempe et al., J. Am. Chem. Soc., 118, 7083 (1996)], POEPS [Renil et al., Tetrahedron Lett., 36, 4647 (1995)], POEPOP [Renil et al., Tetrahedron Lett., 34, 6185 (1996)] and SPOCC [Rademann et al., J. Am. Chem. Soc., 121, 5459 (1999)] resins were developed and tested successfully for solid phase synthesis. One of the limitations of these resins is the imbalance of extent of swelling and functional group capacity.

Summary of the Invention

The present invention provides an efficient, high capacity resin for the solid phase synthesis, immobilization and chromatographic separation as specific applications.

The present invention in one aspect is directed to novel poly(alkylene)glycol based high loading, high swelling polymer beads with a high mechanical and chemical sta

bility. The polymer matrices according to the invention can be prepared from poly(alkylene)glycol acrylamide resins by the exhaustive reduction of amide groups.

The polymer matrices according to the present invention is highly flow stable and polar which assists the peptide solvatization, allowing the diffusion of polar components into the interior of the beads. Also, the polymer beads are transparent with no absorbance in the aromatic region to allow the spectroscopic monitoring of reaction within the resin. The polymer is highly swellable in various solvents and there is no considerable change in the swelling property even after a series of reactions performed on the resin. Also the density of the resin permit multiple column peptide synthesis.

The present invention in one preferred aspect provides a beaded or granulated polymer matrix formed by reduction of a substantial amount of amide groups, such as more than 50% of amide groups, in a cross-linked polymer obtainable by polymerization of a poly(oxyalkylene) chain terminated with an acylamide functional group using radical or ionic initiators.

The cross-linked polymer can be obtained by polymerization of a poly(oxyalkylene) chain terminated with an acylamide functional group using radical and/or ionic initiators, and the beading can be achieved by inverse suspension polymerization

In one embodiment, the cross-linked polymer is obtained by a method comprising the steps of

i) providing a plurality of macromonomers each comprising a poly(oxyalkylene) chain terminated with an acylamide functional group,

ii) polymerising said macromonomer using a free radical initiator or an ionic initiator, optionally with the addition of copolymerizing agents, and

iii) converting in the beaded polymer matrix more than 50% of the amide functional groups to amine functional groups by reduction of the amide functional groups with a suitable reducing agent.

The polymerization can be achieved by e.g. inverse suspension polymerization leading to the formation of a beaded resin, or a granulated matrix can be obtained.

Definitions

Beaded polymer matrix: A beaded polymer matrix is a multitude of beads of crosslinked polymer formed by beading according to principles of suspension or inverse suspension polymerization, by spray polymerization, or by droplet polymerization.

Substantial amount of amide groups in a resin: At least more than 50%, such as more than 60%, for example more than 70%, such as more than 80%, for example more than 90%, such as more than 95%, for example more than 99% of the amide groups present in the resin.

Solid phase synthesis: Synthesis where one of several of the reactants forming the target molecule is attached to a solid support e. g. a beaded polymer

Swelling: When beads or granules or particles are capable of swelling, any physical measurement of the afore-mentioned, including size determinations and volume determinations, refer to measurements conducted for the swelled bead or granule or particle. Swelling of the beads are for practical reasons measured as the volume of a packed bed of beads swollen in a specific solvent and divided by the dry weight of the beads. The unit is given as ml/g. Typical solvents are water, methanol and dichloromethane, but any suitable solvent may be chosen.

Degree of polymerization: The number of monomeric units in a macromolecule or oligomeric molecule, a block or a chain.

Brief Description of the Drawings

Fig. 1: Synthesis of high capacity poly(alkylene)glycol-based resin (Resin B) from poly(alkylene)glycol acrylamide resin (Resin A)

Fig. 2: Optical micrograph of the high capacity poly(ethylene)glycol-based resin (Resin B)

Fig. 3: Swelling character of high capacity poly(ethylene)glycol-based resin (Resin B) and PEGA (Resin A) in various solvents

Fig. 4: IR spectroscopy of (a) PEGA (Resin A) and (b) high capacity poly(ethylene)glycol based resin (Resin B)

Fig. 5: Stability comparison of high capacity poly(ethylene)glycol based resin (Resin B) by IR spectroscopy after treatment with various reagents (a) original (b) 20% Piperidine/DMF (c) Saturated aq. NaOH (d) DBU (100%) (e) triflic anhydride (100%) (f) BF_3 Et_2O (100%) (g) BuLi (2.7 M solution in heptane, 100%) and (h) TFA (100%)

Fig. 6: Swelling character of the high capacity poly(ethylene)glycol-based resin (Resin B) after 7 days treatment with various reagents

Fig. 7: Mechanical testing studies (a) high capacity poly(ethylene)glycol based resin (Resin B) (b) PEGA resin (Resin A)

Fig. 8: Synthesis of Ac-His-(D)Phe-Arg-Trp-NH_2 [Peptide (1)]

Fig. 9: Synthesis of Fmoc-Dap(N_3)

Fig. 10: Synthesis of cyclic peptidomimetic (3)

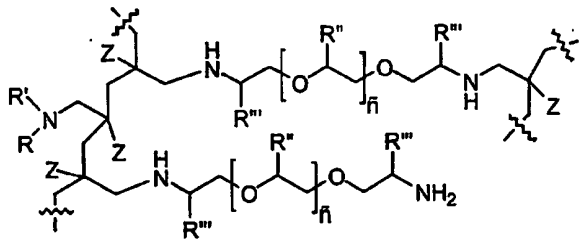
Fig. 11: High performance liquid chromatogram of pure peptide (1)

Fig. 12: High performance liquid chromatogram of pure linear peptidomimetic (2)

Fig. 13: High performance liquid chromatogram of pure cyclic peptidomimetic (3)

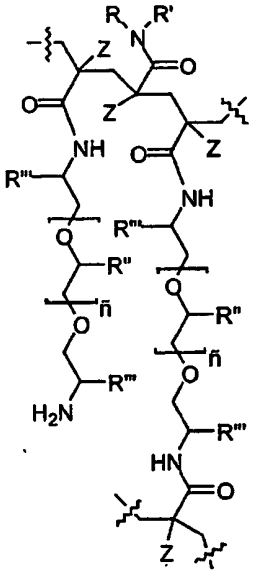
Detailed Description of the Invention

The polymer matrix according to one embodiment of the invention is illustrated as formula 1 herein below. The polymer matrix can be prepared by exhaustive reduction of amide groups in the polymer matrix of formula 2.



Z = H or CH_3 or C_2H_5
R = H or CH_3 or CH_2OH or C_2H_5OH or i-C_3H_7 or n-C_3H_7 or i-C_4H_9 or n-C_4H_9
R' = H or CH_3 or i-C_3H_7 or n-C_3H_7 or i-C_4H_9 or n-C_4H_9
R" = H or CH_3
R"' = H or CH_3

(1)



Z = H or CH_3 or C_2H_5
R = H or CH_3 or CH_2OH or C_2H_5OH or i-C_3H_7 or n-C_3H_7 or i-C_4H_9 or n-C_4H_9
R' = H or CH_3 or i-C_3H_7 or n-C_3H_7 or i-C_4H_9 or n-C_4H_9
R" = H or CH_3
R"' = H or CH_3

(2)

wherein ñ is a real number and designates the average degree of polymerization (dp) of poly(alkylene)glycol in the range of from 3 to 2000.

ñ can be a real number in the range of from 3 to 2000, such as from 3 to 800, for example from 3 to 600, such as from 3 to 400, for example from 3 to 300, for exam

ple from 3 to 200, such as from 3 to 100, for example from 3 to 90, such as from 3 to 80, for example from 3 to 70, such as from 3 to 60, for example from 3 to 50, such as from 3 to 45, for example from 3 to 40, such as from 3 to 30, for example from 3 to 25, such as from 10 to 25, for example from 10 to 20, such as from 10 to 15, for example from 15 to 20, such as from 11 to 19, for example from 12 to 18, such as from 13 to 17, for example from 10 to 12, such as from 12 to 14, for example from 14 to 16, such as from 16 to 18, for example from 18 to 20.

A preferred value for n is between about 4 and about 180, such as about 10, for example about 20, such as about 30, for example about 40, such as about 50, for example about 60, such as about 70, for example about 80, such as about 90, for example about 100, such as about 110, for example about 120, such as about 130, for example about 140, such as about 150, for example about 160, such as about 170, i.e. the compound is preferably a derivative of PEG_{194} to PEG_{8000} or of PPG_{460} to PPG_{4800}.

The polymer matrices according to the invention can be obtained by the reduction of a polymer obtained by the polymerization of a 50-100% partially or fully acryloylated O,O`-bisaminoprop-1-yl)PEG_{1900} and 0-50% acrylamide by weight, with the preferred amount of acrylamide being less than 15%.

The polymer matrix employed in the invention comprises or consists of insoluble, crosslinked poly(alkylene)glycols. The polymer is highly crosslinked and suitable for use in a wide range of applications. The polymer of the present invention can be used as a solid support in a range of solid phase synthetic applications, as a stationary phase in chromatography, and matrices for immobilization of macromolecules. The polymers of the present invention can be synthesized in a wide range of molecular weights and crosslinking. The polymer can be homopolymers or copolymers and can be substituted or unsubstituted.

The polymer can be a homopolymer or a copolymer of one or more amine containing monomers in combination with one or more non-amine containing monomers. The polymer is prepared from monomers either by bulk, suspension or inverse suspension polymerization techniques. Examples of non-amine containing monomers include vinyl alcohol such as vinyl benzyl alcohol; vinyl carboxylic acids such as

acrylic acid, methacrylic acid, itaconic acid, and vinyl benzoic acid; vinyl esters such as vinyl acetate, vinyl propionate; allyl esters such as allyl acetate; allyl amines such as allyl ethyl amine; allyl alcohols such as allyl alcohol, 1-buten-3-ol, 1-penten-3-ol, 1-hexen-3-ol, 1-hydroxy-1-vinyl cyclohexane, 2-bromoallyl alcohol, 2-chloroallyl alcohol; hydroxy containing vinyl ethers such as hydroxyethyl vinyl ether; vinyl acid halides such as acryloyl chloride and methacryloyl chloride; styrenes and substituted styrenes such as 4-ethyl styrene, 4-amino styrene, dichlorostyrene, chlorostyrene, 4-hydroxystyrene, hydroxymethyl styrene, and 4-hydroxy-3-nitro styrene; vinyl toluene; hetroaromatic vinyl such as 1-vinylimidazole, 4-vinyl pyridine, and 2-vinyl pyridine; acrylamide; dimethyl acrylamide; and hydroxy containing (meth)acrylamides such as N-(hydroxymethyl) (meth)acrylamide, N-(1-hydroxyethyl) (meth)acrylamide, N-methyl-N-(2-hydroxyethyl) (meth)acrylamide, N-(1-hexyl-2-hydroxy-1-methylethyl) (meth)acrylamide, N-propyl-N-(2-hydroxyethyl) (meth)acrylamide. Examples of amine containing monomers preferably bisamino polyethyleneglycol with various chain length. Most preferably the polymer is a copolymer of bisamino polyethyleneglycol with acrylamide, such as a high-density amine functionality is accessible after the exhaustive reduction.

Preferably the polymer is insoluble by crosslinking. The cross-linking agent can be characterized by functional groups, which react with amino group of the bisamino poly(alkylene)glycol. Alternatively, the crosslinking group can be characterized by vinyl groups, which can be polymerized by free radical polymerization with amine monomer.

The level of crosslinking makes the polymer differ in their swelling behaviour, which directly affect the reactivity of the polymer. The free radical initiators useful in the present invention include azo compounds, tertiary amines, organic and inorganic peroxides and peroxodisulphates. The preferred free radical initiator is ammonium peroxodisulfate. The commercial products include VAZO 67, VAZO 64 and VAZO 52 can also be used as the initiator.

The number average molecular weight (M_n) of the polymer matrix is in the range of from 200 to 60000, such as from 200 to 45000, for example from 200 to 30000, such as from 200 to 25000, for example from 200 to 20000, such as from 200 to 15000, for example from 200 to 10000, such as from 200 to 8000, for example from 200 to

6000, such as from 200 to 5000, for example from 200 to 4500, such as from 200 to 4000, for example from 200 to 3500, such as from 200 to 3000, for example from 200 to 2500, such as from 200 to 2400, for example from 200 to 2300, such as from 200 to 2200, for example from 200 to 2100, such as from 200 to 2000, for example from 200 to 1900, such as from 200 to 1800, for example from 200 to 1700, such as from 200 to 1600, for example from 200 to 1500, such as from 200 to 1400, for example from 200 to 1300, such as from 200 to 1200, for example from 200 to 1100, such as from 200 to 1000, for example from 200 to 900, such as from 200 to 800, for example from 200 to 700, such as from 200 to 600, for example from 400 to 3000, such as from 400 to 2500, for example from 400 to 2000, such as from 400 to 1800, for example from 400 to 1600, such as from 400 to 1400, for example from 400 to 1200, such as from 400 to 1000, for example from 400 to 800, such as from 400 to 600, for example from 600 to 3000, such as from 600 to 2500, for example from 600 to 2000, such as from 600 to 1800, for example from 600 to 1600, such as from 600 to 1400, for example from 600 to 1200, such as from 600 to 1000, for example from 600 to 800, such as from 800 to 3000, for example from 800 to 2500, such as from 800 to 2000, for example from 800 to 1800, such as from 800 to 1600, for example from 800 to 1400, such as from 800 to 1200, for example from 800 to 1000, such as from 1000 to 3000, for example from 1000 to 2500, such as from 1000 to 2000, for example from 1000 to 1800, such as from 1000 to 1600, for example from 1000 to 1400, such as from 1000 to 1200.

The amine group loading capacity of the polymer matrix is preferably in the range of from 0.01 to 14 mmol/gram, such as from 0.01 to 13 mmol/gram, for example from 0.01 to 12 mmol/gram, for example from 0.01 to 11 mmol/gram, such as from 0.01 to 10 mmol/gram, for example from 0.01 to 9 mmol/gram, such as from 0.01 to 8 mmol/gram, for example from 0.01 to 7 mmol/gram, such as from 0.01 to 6 mmol/gram, for example from 0.01 to 5 mmol/gram, such as from 0.01 to 4 mmol/gram, for example from 0.01 to 3 mmol/gram, such as from 0.01 to 2 mmol/gram, for example from 0.01 to 1 mmol/gram, such as from 0.01 to 0.5 mmol/gram, for example from 0.01 to 0.4 mmol/gram, such as from 0.02 to 2 mmol/gram, for example from 0.04 to 2 mmol/gram, such as from 0.06 to 2 mmol/gram, for example from 0.08 to 2 mmol/gram, such as from 0.1 to 13 mmol/gram, for example from 0.1 to 12 mmol/gram, such as from 0.1 to 11 mmol/gram, for example from 0.1 to 10 mmol/gram, such as from 0.1 to 5

mmol/gram, for example from 0.1 to 4 mmol/gram, such as from 0.1 to 3 mmol/gram, for example from 0.1 to 2 mmol/gram, such as from 0.1 to 1.5 mmol/gram, for example from 0.1 to 1 mmol/gram, such as from 0.1 to 0.8 mmol/gram, for example from 0.1 to 0.6 mmol/gram, such as from 0.1 to 0.5 mmol/gram, for example from 0.1 to 0.4 mmol/gram, such as from 0.1 to 0.3 mmol/gram, for example from 0.1 to 0.2 mmol/gram, such as from 0.2 to 2 mmol/gram, for example from 0.4 to 2 mmol/gram, such as from 0.6 to 2 mmol/gram, for example from 0.8 to 2 mmol/gram, such as from 0.9 to 2 mmol/gram, for example from 1.5 to 2 mmol/gram, such as from 0.4 to 1.3 mmol/gram, for example from 0.6 to 1.3 mmol/gram, such as from 0.8 to 1.3 mmol/gram, for example from 1 to 2 mmol/gram, such as from 1.2 to 2 mmol/gram, for example from 1.4 to 2 mmol/gram, such as from 1.6 to 2 mmol/gram, for example from 1.8 to 2 mmol/gram, such as from 0.01 to 0.05 mmol/gram, for example from 0.05 to 0.1 mmol/gram, such as from 0.1 to 0.2 mmol/gram, for example from 0.2 to 0.4 mmol/gram, such as from 0.4 to 0.6 mmol/gram, for example from 0.6 to 0.8 mmol/gram, such as from 0.8 to 1 mmol/gram, such as from 1 to 1.2 mmol/gram, for example from 1.2 to 1.4 mmol/gram, such as from 1.4 to 1.6 mmol/gram, for example from 1.6 to 1.8 mmol/gram.

The swelling volume of the polymer matrix in an aqueous liquid, including water, is in the range of from 1 ml/gram to preferably less than 32 ml/gram, such as from 1 ml/gram to 24 ml/gram, for example from 1 ml/gram to 20 ml/gram, such as from 1 ml/gram to 18 ml/gram, for example from 1 ml/gram to 16 ml/gram, such as from 1 ml/gram to 14 ml/gram, for example from 1 ml/gram to 12 ml/gram, such as from 1 ml/gram to 10 ml/gram, for example from 1 ml/gram to 9 ml/gram, such as from 1 ml/gram to 8 ml/gram, for example from 1 ml/gram to 7 ml/gram, such as from 1 ml/gram to 6 ml/gram, for example from 1 ml/gram to 5 ml/gram, such as from 1 ml/gram to 4 ml/gram, for example from 1 ml/gram to 3 ml/gram, such as from 1 ml/gram to 2 ml/gram, for example from 4 ml/gram to 20 ml/gram, such as from 4 ml/gram to 18 ml/gram, for example from 4 ml/gram to 16 ml/gram, such as from 4 ml/gram to 14 ml/gram, for example from 4 ml/gram to 12 ml/gram, such as from 4 ml/gram to 10 ml/gram, for example from 4 ml/gram to 8 ml/gram, such as from 4 ml/gram to 6 ml/gram, for example from 6 ml/gram to 20 ml/gram, such as from 6 ml/gram to 18 ml/gram, for example from 6 ml/gram to 16 ml/gram, such as from 6 ml/gram to 14 ml/gram, for example from 6 ml/gram to 12 ml/gram, such as from 6

ml/gram to 10 ml/gram, for example from 6 ml/gram to 8 ml/gram, such as from 8 ml/gram to 20 ml/gram, for example from 8 ml/gram to 16 ml/gram, such as from 8 ml/gram to 12 ml/gram, for example from 2 ml/gram to 4 ml/gram, such as from 8 ml/gram to 10 ml/gram, for example from 10 ml/gram to 12 ml/gram, such as from 12 ml/gram to 14 ml/gram, for example from 14 ml/gram to 16 ml/gram, such as from 16 ml/gram to 18 ml/gram, for example from 18 ml/gram to 20 ml/gram.

The ratio R between i) the amine group loading capacity and ii) the swelling volume of the matrix in an aqueous liquid, such as e.g. water, is in the range of from 10^{-4} to 0.5, such as from 10^{-4} to 0.4, for example from 10^{-4} to 0.3, such as from 10^{-4} to 0.2, for example from 10^{-4} to 0.1, such as from 10^{-4} to 0.09, for example from 10^{-4} to 0.08, such as from 10^{-4} to 0.07, for example from 10^{-4} to 0.06, such as from 10^{-4} to 0.05, for example from 10^{-4} to 0.04, such as from 10^{-4} to 0.03, for example from 10^{-4} to 0.02, such as from 10^{-4} to 0.01, for example from 10^{-4} to 0.009, such as from 10^{-4} to 0.005, for example from 10^{-3} to 0.5, such as from 10^{-3} to 0.4, for example from 10^{-3} to 0.3, such as from 10^{-3} to 0.2, for example from 10^{-3} to 0.1, such as from 10^{-3} to 0.09, for example from 10^{-3} to 0.08, such as from 10^{-3} to 0.06, for example from 10^{-3} to 0.04, such as from 0.01 to 0.5, for example from 0.1 to 0.5, such as from 0.01 to 0.4, for example from 0.02 to 0.04, such as from 0.04 to 0.08, for example from 0.05 to 0.5, such as from 0.08 to 0.5.

The polymer matrix can be beaded or granulated. When the polymer matrix is beaded, it has an essentially spherical form, and preferably a diameter in the range of from 0.1 μm to preferably less than 3000 μm. A more preferred range of diameter is between 10 μm and 1000 μm.

The beaded, cross-linked polymer matrix can be formed by polymerization of droplets in an inert phase, such as unreactive oil, for example paraffin oil. The polymer resin can also be formed by bulk polymerization followed by granulation.

There is also provided a composition comprising a plurality of cross-linked polymer beads according to the invention. The composition preferably comprises more than 10^3 beads, for example more than 10^5 beads, such as more than 10^7 beads, for example more than10^9 beads.

The average diameter of the beads of the composition is preferably in the range of from 0.1 μm to less than 3000 μm.

In a further aspect there is provided a functional surface comprising the polymer matrix according to the invention, obtained e.g. by bulk or moulded polymerization, and attached thereto at least one functional moiety. The functional moiety can be a bioactive species preferably selected from a scaffold moiety comprising at least one site for functionalization, a RNA moiety, a DNA moiety, a peptide moiety, or an amino acid residue. The functional surface can be planar, tubular, spherical or a porous material.

The functional surface can further comprise a linker residue linking the functional moiety to the functional surface.

Methods for generating the polymer matrix according to the invention

There is provided a method for generating a beaded polymer matrix according to the invention, said method comprising the steps of

synthesizing a monomer and/or macromonomer and a crosslinker for polymerization, and,

polymerizing the macromonomer by either i) suspension polymerization and/or; ii) inverse suspension polymerization and/or iii) bulk polymerization followed by granulation and/or iv) droplet polymerization and/or v) emulsion polymerization and/or vi) seeded polymerization,

and obtaining a polymer matrix according to the invention.

The polymerization reaction can be a radical initiated chain polymerization reaction as disclosed by Meldal in US 5,352,756.

In preferred embodiments, there is provided a method for preparing a beaded polymer matrix according to the invention, said method comprising the steps of

providing a macromonomer comprising a bisamino poly(alkylene)glycol functionalized with at least one fragment comprising a conjugated vinyl group,

mixing the conjugated vinyl macromonomer with acrylic amide derivatives,

copolymerizing the vinyl groups of said macromonomers using radical initiators or ionic initiators,

forming a beaded, cross-linked polymer matrix comprising a plurality of amide functionalities,

reducing the amide functionalities, and

obtaining a beaded polymer matrix wherein the majority of the amide functionalities are reduced to primary and secondary amine functionalities.

The exhaustive reduction of amide groups in the above described polymers can be achieved by the treatment with reducing agents like borane-THF reagent in presence of boric acid and trimethyl borate, arsenic trioxide in aqueous alcoholic HCl, antimony pentoxide in aqueous alcoholic HCl, $LiAlH_4$, H_2O_2, $BF_3.Et_2O$ in presence of sodium borate, lithium tri-(tert)-butoxyaluminium hydride ($LiAlH(OtBu)_3$), DIBAL-H, $NaBH_4$, $NaBH_3CN$ and NaH . The preferred reducing agent for the amide groups are borane-THF reagent in presence of boric acid and trimethyl borate [Yu et al, J. Org. Chem., 67,3138 (2002)]. The reaction has been optimized by using different concentration of reagent cocktail such as (i) borane-THF (2 equiv)/boric acid (1 equiv)/trimethyl borate (1 equiv); (ii) borane-THF (5 equiv)/boric acid (1.5 equiv)/trimethyl borate (1.5 equiv); (iii) borane-THF (7 equiv)/boric acid (2.5 equiv)/trimethyl borate (2.5 equiv); (iv) borane-THF (10 equiv)/boric acid (3 equiv)/trimethyl borate (3 equiv); (v) borane-THF (20 equiv)/boric acid (6 equiv)/trimethyl borate (6 equiv); (vi) borane-THF (40 equiv)/boric acid (12 equiv)/trimethyl borate (12 equiv). A quantitative amide bond reduction to amine is observed in all reactions except for the reactions using less than 10 equiv of reducing agent.

Applications of the polymer matrix

The polymer of the present invention can be used a solid support for a range of applications including solid phase synthesis, chromatography and immobilization of macromolecules.

The polymers according to the invention may again be derivatized with any of the commercial available linkers for solid phase synthesis, such as e.g. linkers comprising functional groups comprising or consisting of one or more of amino, alkylamino, hydroxy, carboxyl, mercapto, sulfeno, sulfino, sulfo, and derivatives of these. The resin can also be used for the combinatorial library synthesis. The resin is also suitable for syntheses involving enzymatic reactions.

The invention also relates to a solid support for enzymatic synthesis of oligosaccharides with glycosyltransferases said support including a polymer according to the invention as described above.

The invention also relates to a solid support for the immobilization of proteins said support involving a polymer according to the invention as described above.

Again, the invention relates to a resin for chromatographic separations such as affinity chromatography, size exclusion chromatography, ion exchange chromatography, ion pair chromatography, normal phase chromatography and reversed phase chromatography said resin involving a polymer according to the invention as described above.

The invention also relates to a method of continuous flow or batchwise synthesis of peptides, oligonucleotides or oligosaccharides during the synthesis is attached to a solid support involving a polymer according to the invention as described above. Due to the particular features of the polymer according to the invention this method also can extend to the synthesis involving enzymatic reactions.

The invention relates to a method of immobilizing a protein wherein a protein is attached to a solid support involving a polymer according to the invention as described above.

The invention also relates to a method of performing chromatographic separations which comprises the use of a chromatographic resin involving a polymer according to the invention as described above.

Further, the invention relates to a solid support for scavenging the excess reagents in solution phase synthesis involving a polymer according to the invention as described above. In preferred embodiments, there is provided the use of the polymer matrix according to the invention for scavenging excess acyl compounds or excess carbonyl compounds from a composition comprising a mixture of molecular entities.

Also provided is a partially acryloylated bisamino poly(alkylene)glycol for use in the preparation of a beaded, cross-linked polymer matrix according to the invention, said preparation preferably comprising the step of inverse suspension polymerization.

There is also provided the following methods:

A method for preparing a functional surface said method comprising the steps of

i) providing a macromonomer comprising a bisamino poly(alkylene)glycol functionalized with at least one fragment comprising a vinyl group,

ii) mixing the conjugated vinyl macromonomer with acrylic amide derivatives

iii) polymerizing the vinyl groups of said macromonomers using radical initiators or ionic initiators,

iv) forming a beaded, cross-linked polymer matrix comprising a plurality of amide functionalities,

v) reducing the amide functionalities,

vi) obtaining a beaded polymer matrix wherein the majority of the amide functionalities are reduced to primary and secondary amine functionalities, and

vii) contacting the beaded polymer matrix obtained in step e) with at least one functional moiety and obtaining the functional surface.

A method for targeting a functional moiety attached to a functional surface, said method comprising the steps of

i) providing a functional surface according to the invention, and

ii) targeting said functional moiety with at least one targeting species having a non-covalent affinity, for said functional moiety, or

iii) targeting said functional moiety with at least one targeting species forming a covalent bond with the said functional moiety.

A method for identifying and/or purifying a targeting species having an affinity for a functional moiety, said method comprising the steps of

i) providing a functional surface according to the invention, and

ii) targeting said functional moiety with at least one targeting species having an affinity for said functional moiety, and

iii) identifying and/or purifying the at least one targeting species having an affinity for said functional moiety.

Targeting species identified and/or purified by the above method are also within the scope of the invention as are methods for therapy of a human or animal body when said methods comprise the step of administering to said human or animal body a targeting species identified as described herein above in a pharmaceutical effective amount.

There is also provided the use of the polymer matrix according to the invention as a support for combinatorial chemistry reactions and the synthesis of combinatorial libraries.

Accordingly, one preferred application for the polymer resins according to the invention is in the synthesis of libraries using combinatorial chemistry. A combinatorial library is a collection of multiple species of chemical compounds comprised of smaller subunits or monomers. Combinatorial libraries come in a variety of sizes, ranging from a few hundred to many hundreds of thousand different species of chemical compounds. There are also a variety of library types, including oligomeric and polymeric libraries comprised of compounds such as peptides, carbohydrates, oligonucleotides, and small organic molecules, etc. Such libraries have a variety of uses, such as immobilization and chromatographic separation of chemical compounds, as well as uses for identifying and characterizing ligands capable of binding an acceptor molecule or mediating a biological activity of interest.

The library compounds may comprise any type of molecule of any type of subunits or monomers, including small molecules and polymers wherein the monomers are chemically connected by any sort of chemical bond such as covalent, ionic, coordination, chelation bonding, etc., which those skilled in the art will recognize can be synthesized on a solid-phase support

The term polymer as used herein includes those compounds conventionally called heteropolymers, i.e., arbitrarily large molecules composed of varying monomers, wherein the monomers are linked by means of a repeating chemical bond or structure. The polymers of the invention of this types are composed of at least two subunits or monomers that can include any bi-functional organic or herteronuclear molecule including, but not limited to amino acids, amino hydroxyls, amino isocyanates, diamines, hydroxycarboxylic acids, oxycarbonylcarboxylic acids, aminoaldehydes, nitroamines, thioalkyls, and haloalkyls.

In the disclosure of the present invention, the terms "monomer," "subunits" and "building blocks" will be used interchangeably to mean any type of chemical building block of molecule that may be formed upon a solid-phase support. The libraries are not limited to libraries of polymers, but is also directed to libraries of scaffolded small molecules.

Various techniques for synthesizing libraries of compounds on solid-phase supports are known in the art. Solid-phase supports are typically polymeric objects with sur

faces that are functionalized to bind with subunits or monomers to form the compounds of the library. Synthesis of one library typically involves a large number of solid-phase supports.

To make a combinatorial library, solid-phase supports are reacted with a one or more subunits of the compounds and with one or more numbers of reagents in a carefully controlled, predetermined sequence of chemical reactions. In other words, the library subunits are "grown" on the solid-phase supports. The larger the library, the greater the number of reactions required, complicating the task of keeping track of the chemical composition of the multiple species of compounds that make up the library. Thus, it is important to have methods and apparatuses which facilitate the efficient production of large numbers of chemical compounds, yet allow convenient tracking of the compounds over a number of reaction steps necessary to make the compounds.

Combinatorial libraries represent an important tool for the identification of e.g. small organic molecules that affect specific biological functions. Due to the interaction of the small molecules with particular biological targets and their ability to affect specific biological functions, they may also serve as candidates for the development of therapeutics. Accordingly, small molecules can be useful as drug leads eventually resulting in the development of therapeutic agents.

Because it is difficult to predict which small molecules will interact with a biological target. Intense efforts have been directed towards the generation of large numbers, or "libraries", of small organic compounds. These libraries can then be linked to sensitive screens to identify the active molecules.

Libraries have been designed to mimic one or more features of natural peptides. Such peptidomimetic libraries include phthalimido libraries (WO 97/22594), thiophene libraries (WO 97/40034), benzodiazopene libraries (U.S. Pat. No. 5,288,514), libraries formed by sequential reaction of dienes (WO 96/03424), thiazolidinone libraries, libraries of metathiazanones and their derivatives (U.S. Pat. No. 5,549,974), and azatide libraries (WO 97/35199) (for peptidomimetic technologies, see Gante, J., Angew. Chem. Int. Ed. Engl. 1994, 33, 1699-1720 and references cited therein).

Examples

General Methods

Reagents were obtained from Aldrich and used without any purification. All solvents used were of HPLC grade kept over molecular sieves. The PEGA beads were prepared in a 250 ml baffled glass reactor equipped with a dispersion stirrer.

Synthesis of partially acryloylated $(NH_2)_2PEG_{1900}$

Acryloyl chloride (1.267 ml, 14 mmol) in DCM (12 ml) was added dropwise to a solution of $(NH_2)_2PEG_{1900}$ (20 g, 10 mmol) in DCM (18 ml) at 0 °C with stirring. The reaction mixture was kept for 1 h at 20 °C. The DCM was evaporated and drying in vacuo at 20 °C yielded the 70% acyloylated $(NH_2)_2PEG_{1900}$ as colourless thick oil.

Preparation of $PEGA_{1900}$ beads (Resin A)

The $PEGA_{1900}$ polymer beads were prepared by inverse suspension polymerization method. In order to prepare the beads having a size 500 μm, a 1.4 wt % of sorbitan monolaurate with the macromonomer was used as the suspension stabilizer. The n-heptane was used as the suspension medium and was degassed with argon for 1 h before the addition of monomers. In a typical synthesis procedure, a solution of $(Acr)_{1.4}$ $(NH_2)_2PEG_{1900}$ (7.3 g, 3.54 mmol) in water (21 ml) was degassed with argon for 30 min. Acrylamide (0.36 g, 5 mmol) in water (0.5 ml) was added to the degassed solution and the purging of argon was continued for 5 min. A solution of sorbitan monolaurate (0.1 ml) in DMF (1 ml) and the free radical initiator ammonium persulfate (300 mg) in water (2 ml) were added to the monomer mixture. The reaction mixture was then rapidly added to the suspension medium and stirred at 600 rpm at 70 °C. After one min, TEMED (1.5 ml) was added to the reactor. The reaction was allowed to continue for 3h, the beads formed were filtered through the sieves and the 500 μm fraction was collected. The beads were washed thoroughly with ethanol (10×), water (10×), ethanol (10×) and dried under high vacuum to provide Resin A.

Synthesis of high loading poly(ethylene)glycol based resin (Resin B) by exhaustive reduction

The exhaustive reduction of amide groups in PEGA resin (Resin A) was carried out in a 100 ml glass tubes under argon.

a) The resin (500 mg, 0.85 mmol carbonyl) and boric acid (1 equiv, 52.55 mg, 0.85 mmol) were taken in the glass tube. Trimethyl borate (1 equiv, 100 µl, 0.85 mmol) was added followed by the addition of 1M borane-THF complex (2 equiv, 1.7 ml, 1.7 mmol). After cessation of hydrogen evolution, the tubes were capped tightly and kept in an oil bath at 65°C for 72 h. The resin was then filtered, washed with DMF (10 ml × 4) and MeOH (10 ml × 4). The resin was then suspended in piperidine (100%, 10 ml) and heated at 65°C for 20 h to disproportionate the borane complexes. Following the decantation of the piperidine-borane solution, the resin was washed with DMF (10 ml × 4), DCM (10 ml × 4) and MeOH (10 ml × 4) and dried under vacuum to provide Resin B with 30 % of amide reduction.

b) The resin (500 mg, 0.85 mmol carbonyl) and boric acid (1.5 equiv, 78.83 mg, 1.27 mmol) were taken in the glass tube. Trimethyl borate (1.5 equiv, 150 µl, 1.27 mmol) was added followed by the addition of 1M borane-THF complex (5 equiv, 4.25 ml, 4.25 mmol). After cessation of hydrogen evolution, the tubes were capped tightly and kept in an oil bath at 65°C for 72 h. The resin was then filtered, washed with DMF (10 ml × 4) and MeOH (10 ml × 4). The resin was then suspended in piperidine (100%, 10 ml) and heated at 65°C for 20 h to disproportionate the borane complexes. Following the decantation of the piperidine-borane solution, the resin was washed with DMF (10 ml × 4), DCM (10 ml × 4) and MeOH (10 ml × 4) and dried under vacuum to provide Resin B with 50 % of amide reduction.

c) The resin (500 mg, 0.85 mmol carbonyl) and boric acid (2.5 equiv, 131.4 mg, 2.13 mmol) were taken in the glass tube. Trimethyl borate (2.5 equiv, 250 µl, 2.13 mmol) was added followed by the addition of 1M borane-THF complex (7 equiv, 5.95 ml, 5.95 mmol). After cessation of hydrogen evolution, the tubes were capped tightly and kept in an oil bath at 65°C for 72 h. The resin was then filtered, washed with DMF (10 ml × 4) and MeOH (10 ml × 4). The resin was then suspended in piperidine (100%, 10 ml) and heated at 65°C for 20 h to disproportionate the borane complexes. Following the decantation of the piperidine-borane solution, the resin was washed with DMF (10 ml × 4), DCM (10 ml × 4) and MeOH (10 ml × 4) and dried under vacuum to provide Resin B with 75 % of amide reduction.

d) The resin (500 mg, 0.85 mmol carbonyl) and boric acid (3 equiv, 157.66 mg, 2.55 mmol) were taken in the glass tube. Trimethyl borate (3 equiv, 300 µl, 2.55 mmol) was added followed by the addition of 1M borane-THF complex (10 equiv, 8.5 ml, 8.5 mmol). After cessation of hydrogen evolution, the tubes were capped tightly and kept in an oil bath at 65°C for 72 h. The resin was then filtered, washed with DMF (10 ml x 4) and MeOH (10 ml x 4). The resin was then suspended in piperidine (100%, 10 ml) and heated at 65°C for 20 h to disproportionate the borane complexes. Following the decantation of the piperidine-borane solution, the resin was washed with DMF (10 ml x 4), DCM (10 ml x 4) and MeOH (10 ml x 4) and dried under vacuum to provide Resin B with a quantitative conversion of amide to amine.

e) The resin (500 mg, 0.85 mmol carbonyl) and boric acid (6 equiv, 315.32 mg, 5.1 mmol) were taken in the glass tube. Trimethyl borate (6 equiv, 600 µl, 5.1 mmol) was added followed by the addition of 1M borane-THF complex (20 equiv, 17 ml, 17 mmol). After cessation of hydrogen evolution, the tubes were capped tightly and kept in an oil bath at 65°C for 72 h. The resin was then filtered, washed with DMF (10 ml x 4) and MeOH (10 ml x 4). The resin was then suspended in piperidine (100%, 10 ml) and heated at 65°C for 20 h to disproportionate the borane complexes. Following the decantation of the piperidine-borane solution, the resin was washed with DMF (10 ml x 4), DCM (10 ml x 4) and MeOH (10 ml x 4) and dried under vacuum to provide Resin B with a quantitative conversion of amide to amine.

f) The resin (500 mg, 0.85 mmol carbonyl) and boric acid (12 equiv, 630.64 mg, 10.2 mmol) were taken in the glass tube. Trimethyl borate (12 equiv, 1.2 ml, 10.2 mmol) was added followed by the addition of 1M borane-THF complex (40 equiv, 34 ml, 34 mmol). After cessation of hydrogen evolution, the tubes were capped tightly and kept in an oil bath at 65°C for 72 h. The resin was then filtered, washed with DMF (10 ml x 4) and MeOH (10 ml x 4). The resin was then suspended in piperidine (100%, 10 ml) and heated at 65°C for 20 h to disproportionate the borane complexes. Following the decantation of the piperidine-borane solution, the resin was washed with DMF (10 ml x 4), DCM (10 ml x 4) and MeOH (10 ml x 4) and dried under vacuum to provide Resin B with a quantitative conversion of amide to amine.

Characterization

Loading:

The amino functional loading was determined from the Fmoc-Gly derivatized Resin B. The resin (3-5 mg) was treated with piperidine-DMF solution (20% v/v, 8 ml) for 30 min. The amino capacity of the resin was calculated from the OD value of piperidine-dibenzofulvene solution at 290 nm. The amino functional loading of Resin B is measured as 1.6 mmol/g where starting with a resin obtained from the reduction of a polymer obtained by the polymerization of a 95% of partially acryloylated O,O`-bisaminoprop-1-yl)PEG_{1900} and 5% acrylamide (Resin A).

Swelling:

The swelling capabilities of the resin in different solvents were determined by the syringe method. In a typical procedure, the resin (100 mg) was taken in a 2 ml syringe fitted with a Teflon filter at the bottom. The solvent was sucked in to the syringe and after 3 h, excess solvent was removed by applying force on the piston. The extent of swelling of the resin in each solvent was determined from the volume of the resin before and after the solvent incubation.

Mechanical stability:

The mechanical stability of the high capacity poly(ethylene)glycol based resin (Resin B) was compared with PEGA resin (Resin A). The compressive properties describe the behaviour of the bead when it is subjected to a compressive stress. The bead (300-500 μm) is placed between compressive plates parallel to the surface and then compressed at a constant rate. The compressive modulus can be calculated from the ratio of compressive stress (the force per area of cross section of the bead at low strain) over compressive strain (ratio of the diameter over original diameter). Compressive modulus of high capacity poly(ethylene)glycol based resin (Resin B) is 0.5 MPa and that of PEGA resin (Resin A) is 0.4 MPa, which indicates that even after the exhaustive reduction, the resin does not change its mechanical properties considerably.

Chemical stability:

The stability studies of the resin were carried out in different reagents like trifluoro acetic acid (100%), 20% piperidine in DMF, 1,8-diazobicyclo[5.4.0]-undec-7-ene (DBU) (100%), butyl lithium (2.7 M solution in heptane, 100%), saturated NaOH, and

BF_3 Et_2O (100%). The resin samples (100 mg) were separately stirred with the reagents. After 48 h, the resin was filtered, washed, dried and IR spectra were recorded and compared with original. The swelling properties of the resin after treatment with the reagents for two weeks were also compared. The resin did not dissolve in any of these conditions and showed no changes in colour or swelling indicating no bond cleavage.

Use of the high capacity PEG-based resin for solid phase synthesis

Synthesis of Ac-His-(D)Phe-Arg-Trp-NH_2 [Peptide (1)]

The peptides were synthesized in a plastic syringe fitted with a Teflon filter at the bottom.

The high capacity poly(ethylene)glycol based resin (35 mg, 0.056 mmol) was swollen in dry DMF (5 ml) and treated with Fmoc-Rink amide linker (90.65 mg, 0.168 mmol, 3 equiv) in presence of TBTU (51.77 mg, 0.224 mmol, 2.88 equiv) and NEM (28.3 µl, 0.224 mmol, 4 equiv). After 3 h at room temperature, the resin was washed with DMF (10×), MeOH (10×), DCM (10×) and dried in vacuo. The resin was negative to Kaiser amine test and a quantitative reaction was observed by measuring the Fmoc group on the resin (5 mg) with 20% Piperidine/DMF solution (8 ml) for 30 min at room temperature.

The resin was swollen in dry DMF (5 ml) and the Fmoc group was removed by 20% Piperidine/DMF (1 ml) for 20 min at room temperature. The resin was washed with DMF (10×) and the amino acids Fmoc-Trp(Boc), Fmoc-Arg(Pmc), Fmoc-(D)Phe and Fmoc-His(Trt) (3 equiv) were attached successively in presence of TBTU (2.88 equiv) and NEM (4 equiv). After the incorporation of all amino acids, the Fmoc protection was removed by 20% piperidine in DMF (1 ml, 20 min) and the resin was washed with DMF (10×). The peptide on the resin was then acetylated with acetic anhydride/pyridine/DMF (2:4:4) (1 ml) and washed with DMF (10×), MeOH (10×), DCM (10×) and dried in vacuo. The peptide was cleaved from the resin by treating with a solution of TFA (90%), water (5%), ethanedithiol (2%), triisopropyl silane (2%) and thioanisole (1%) for 3 h at room temperature. The resin was filtered off and washed with TFA (2 ×) and DCM (2 ×). The combined filtrate was concentrated under vacuum and the peptide was precipitated by ether. The peptide was washed with ether (10 ×) and dried in vacuo to afford 36.93 mg (96%) of pure peptide.

HPLC: t_R = 9.71 min

ESI-MS: calcd $(M+H)^+$ = 686.78 Da; found $(M+H)^+$ = 686.4

MALDI TOF MS: calcd $(M+H)^+$ = 686.78 Da; found $(M+H)^+$ = 686.98

^{1}H NMR (600 MHz, MeOH-d_4): δ = 1.38-1.64 (m, 2H, Arg H^β), 1.10-1.15 (m, 2H, Arg H^γ), 2.00 (s, 3H, Acetyl CH_3), 2.96 (m, 2H Arg H^δ), 3.00-3.09 (m, 2H Phe H^β), 3.24-3.41 (m, 2H Trp H^β), 3.04-3.23 (m, 2H His H^β), 4.01 (m, 1H Arg H^α), 4.73 (m, 1H His H^α), 4.51 (m, 1H Phe H^α), 4.71 (m, 1H Trp H^α), 7.04-7.67 (br 5H Trp ring protons), 7.21, 8.76 (2H, His ring protons), 7.25-7.33 (br, 5H Phe ring protons).

Synthesis of Fmoc-Dap(N_3)OH

Fmoc-Dap-OH (980 mg, 3 mmol) was dissolved in 80% aqueous acetic acid (9 ml) and $CuSO_4.5H_2O$ (15 mg, 0.06 mmol, 0.02 equiv) in water (1 ml) was added. The pH of the solution was adjusted to 9-10 with K_2CO_3. Water (15 ml), MeOH (32 ml) and trifluoromethanesulfonyl azide (6 mmol) in DCM (25 ml) was added and the pH was readjusted to 9-10 with K_2CO_3. The two-phase system was stirred vigorously for 20 h. The layers were separated by addition of DCM and the organic phase was washed with water (2 x 40 ml) and then the combined aqueous phases were acidified with 3 M HCl (aqueous) to a pH 2. The aqueous phase was extracted with DCM (4 x 50 ml) and the combined organic phases were dried over sodium sulfate, filtered and concentrated under vacuo (0.934 g, 88.2%).

HPLC: t_R = 10.08 min

ESI-MS: calcd $(M+H)^+$ = 353.34 Da; found $(M+H)^+$ = 353.1

^{1}H NMR (250 MHz, $CDCl_3$): δ = 3.75 (d, 2H), 4.14-4.9 (t, 1H), 4.36-4.39 (d, 2H), 4.50-4.54 (m, 1H), 5.50-5.54 (2H, NH and OH), 7.22-7.28 (4H, aromatic ring), 7.51-7.54 (d, 2H, aromatic ring), 7.68-7.71 (d, 2H, aromatic ring).

Synthesis of Fmoc-Lys(Boc)-Dap(N_3)-His(Trt)-(D)Phe-Arg(Pmc)-Trp(Boc)-Pra-Met-HMBA-Gly-Resin B

The high capacity poly(ethylene)glycol based resin (150 mg, 0.24 mmol) was swollen in dry DMF (5 ml) and treated with Fmoc-Gly (215 mg, 0.72 mmol, 3 equiv) in presence of TBTU (222 mg, 0.69 mmol, 2.88 equiv) and NEM (121.8 µl, 0.96 mmol, 4 equiv). After 3 h at room temperature, the resin was washed with DMF (10x), MeOH (10x), DCM (10x) and dried in vacuo. The resin was negative to Kaiser amine test and a quantitative reaction was observed by measuring the Fmoc group

on the resin (5 mg) with 20% Piperidine/DMF solution (8 ml) for 30 min at room temperature.

The resin was swollen in dry DMF (5 ml), Fmoc group was removed by 20% Piperidine/DMF and treated with HMBA linker (109.5 mg, 0.72 mmol, 3 equiv) in presence of TBTU (222 mg, 0.69 mmol, 2.88 equiv) and NEM (121.8 µl, 0.96 mmol, 4 equiv). After 3 h at room temperature, the resin was washed with DMF (10×), MeOH (10×), DCM (10×) and dried in vacuo. The resin was negative to Kaiser amine test

The resin was swollen in dry DCM (2 ml), Fmoc-Met (267.5 mg, 0.72 mmol, 3 equiv), MSNT (213.4 mg, 0.72 mmol, 3 equiv) and MeIm (43 µl, 0.54 mmol, 2.25 equiv) were added. After 1 h, the resin was filtered and washed with DCM (10×), MeOH (10×) and DMF (10×). The Fmoc group was removed by 20% Piperidine/DMF (1 ml) for 20 min at room temperature. The resin was washed with DMF (10×) and the amino acids Fmoc-Pra, Fmoc-Trp(Boc), Fmoc-Arg(Pmc), Fmoc-(D)Phe, Fmoc-His(Trt), Fmoc-Dap(N_3) and Fmoc-Lys(Boc) (3 equiv) were attached successively in presence of TBTU (2.88 equiv) and NEM (4 equiv). After the incorporation of all amino acids, the resin was washed with DMF (10×), MeOH (10×), DCM (10×) and dried in vacuo.

Cleavage of linear peptidomimetic (2) (Lys-Dap(N_3)-His-(D)Phe-Arg-Trp-Pra-Met) from the Resin B

The N-terminal Fmoc protection of the peptidyl resin (8.7 mg) was removed by 20% piperidine/DMF solution (2 ml, 30 min) and the resin was washed with DMF (10×), MeOH (10×), DCM (10×) and dried. The resin was treated with a solution of TFA (90%), water (5%), ethanedithiol (2%), triisopropyl silane (2%) and thioanisole (1%) for 3 h at room temperature for removing all the side chain protection groups. The resin was washed with DCM (10×), MeOH (10×) and DCM (10×).

The peptide was cleaved from the resin by treating with 0.1 M NaOH (100 µl) for 2 h at room temperature. The resin was filtered and the filtrate was neutralized with 0.1 M HCl (100 µl) yielding 4.1 mg (96%) of pure peptide.

HPLC: t_R = 10.63 min

ES MS/MS: calcd $(M+H)^+$ = 1112.29 Da; found $(M-N_3)$ = 1068.63 Da

Cyclization of Fmoc-Lys(Boc)-Dap(N_3)-His(Trt)-(D)Phe-Arg(Pmc)-Trp(Boc)-Pra-Met-HMBA-Gly-Resin B

(a) The peptidyl resin (20 mg) was treated with a solution of TFA (90%), water (5%), ethanedithiol (2%), triisopropyl silane (2%) and thioanisole (1%) for 3 h at room temperature for removing all the side chain protection groups. The resin was washed with DCM (10×), MeOH (10×) and DMF (10×). The Fmoc group was removed by 20% Piperidine/DMF (2 ml) and the resin was washed with DMF (10×), MeOH (10×), DCM (10×) and THF (10×). DIPEA (61 µl, 0.35 mmol, 50 equiv) and CuI (2.66 mg, 0.014 mmol, 2 equiv) in THF (300 µl) were added to the resin. The reaction was left for 16 h and then washed with THF, water, DMF, MeOH, DCM and dried in vacuo.

(b) The peptidyl resin (20 mg) was treated with DIPEA (61 µl, 0.35 mmol, 50 equiv) and CuI (2.66 mg, 0.014 mmol, 2 equiv) in THF (300 µl) were added to the resin. The reaction was left for 16 h and then washed with THF, water, DMF, MeOH, DCM and dried in vacuo. A solution of TFA (90%), water (5%), ethanedithiol (2%), triisopropyl silane (2%) and thioanisole (1%) were added to the resin for removing all the side chain protection groups (3 h at room temperature). The resin was washed with DCM (10×), MeOH (10×) and DMF (10×). The Fmoc group was removed by 20% Piperidine/DMF (2 ml) and the resin was washed with DMF (10×), MeOH (10×), DCM (10×) and dried in vacuo.

Cleavage of cyclic peptidomimetic (3) from the Resin B

The resin was treated with 0.1 M NaOH (100 µl) for 2 h at room temperature. The resin was filtered and the filtrate was neutralized with 0.1 M HCl (100 µl).

(a) Yield= 8.1 mg (82.5%)

(b) Yield= 7.8 mg (79%)

HPLC: t_R = 10.89 min

ES MS/MS: calcd $(M+H)^+$ = 1112.29 Da; found $(M+H)^+$ = 1112.56 Da

^{1}H NMR (600 MHz, DMSO-d_6): 1.259-1.273 (m, 2H Arg H^γ), 1.311-1.332 (m, 2H Lys H^γ), 1.508-1.514 (m, 2H Lys H^δ), 1.416-1.616 (m, 2H Arg H^β), 1.650-1.669 (m, 2H Lys H^β), 1.852-1.979 (m, 2H Met H^β), 2.022 (s, 3H Met $-CH_3$), 2.461 (t, 2H Met H^γ), 2.484-2.577 (m, 2H Pra H^β), 2.671-2.848 (m, 2H His H^β), 2.721-2.944 (m, 2H Phe H^β), 2.728-2.734 (t, 2H Lys H^ε), 2.961-3.157 (m, 2H Trp H^β), 2.998-3.004 (m, 2H Arg

H^{δ}), 3.366-3.568 (m, 2H Dap H^{β}), 3.794 (m, 1H Lys H^{α}), 4.282 (m, 1H Arg H^{α}), 4.309 (m, 1H Met H^{α}), 4.417 (m, 1H Pra H^{α}), 4.521 (m, 1H Dap H^{α}), 4.568 (m, 1H Trp H^{α}), 4.584 (m, 1H His H^{α}), 4.662 (m, 1H Phe H^{α}), 7.164-7.239 (br, 5H Phe ring protons), 7.201, 8.211 (2H, His ring protons), 7.447 (s, 1H Arg –NH), 6.955-7.312 (br, 5H Trp ring protons), 8.240 (s, 1H Triazole ring proton), 8.094 (1H Trp amide H), 8.185 (1H Met amide H), 8.213 (1H Phe amide H), 8.250 (1H Pra amide H), 8.329 (1H Arg amide H), 8.408 (1H His amide H), 8.805 (1H Dap amide H), 10.697 (1H Trp ring NH).

Claims

1. A beaded or granulated polymer matrix formed by a reduction of more than 50% of the amide groups in a cross-linked polymer obtainable by polymerization of a poly(oxyalkylene) chain terminated with an acylamide functional group.

2. The polymer matrix according to claim 1, wherein the cross-linked polymer is obtained by polymerization of a poly(oxyalkylene) chain terminated with an acylamide functional group using radical or ionic initiators.

3. The polymer matrix according to any of claims 1 and 2, wherein the cross-linked and beaded polymer is obtained by a method comprising the steps of

   a) providing a plurality of macromonomers each comprising a poly(oxyalkylene) chain terminated with an acylamide functional group,

   b) polymerising said macromonomer using a free radical initiator or an ionic initiator, optionally with the addition of copolymerizing agents, and

   c) converting in the beaded polymer matrix at least 50% of the amide groups to amine functional groups by reduction of the amide groups with a suitable reducing agent.

4. The polymer matrix according to claim 3, wherein the polymerization is an inverse suspension polymerization.

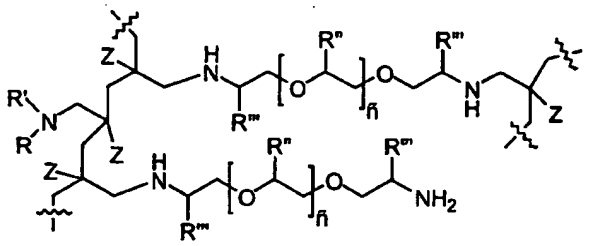
5. The polymer matrix according to any of claims 3 and 4 wherein a free radical initiator is used.

6. The polymer matrix according to any of claims 3 an 4 wherein a ionic initiator is used.

7. The polymer matrix according to any of claims 1 to 6, wherein the reduction of amide groups is achieved by using a reducing agent selected from the group consisting of borane, arsenic trioxide in aqueous alcoholic HCl, antimony pen

toxide in aqueous alcoholic HCl, $LiAlH_4$, H_2O_2, $BF_3.Et_2O$ in the presence of sodium borate, lithium tri-(tert)-butoxyaluminium hydride ($LiAlH(OtBu)_3$), DIBAL-H, $NaBH_4$, $NaBH_3CN$ and NaH.

8. The polymer matrix according to any of claims 1 to 7 comprising the structure



Z = H or CH_3 or C_2H_5
R = H or CH_3 or CH_2OH or C_2H_5OH or i-C_3H_7 or n-C_3H_7 or i-C_4H_9 or n-C_4H_9
R' = H or CH_3 or i-C_3H_7 or n-C_3H_7 or i-C_4H_9 or n-C_4H_9
R" = H or CH_3
R"' = H or CH_3

ñ is a real number in the range of from 3 to 2000

9. The polymer matrix according to claim 8, wherein
Z is selected from H, CH_3 and C_2H_5, and independently thereof,
R is selected from H, CH_3, CH_2OH, C_2H_5OH, i-C_3H_7, n-C_3H_7, i-C_4H_9 and n-C_4H_9, including any combination thereof, and independently thereof,
R` is selected from H, CH_3, i-C_3H_7, n-C_3H_7, i-C_4H_9 and n-C_4H_9, including any combination thereof, and independently thereof,
R`` is selected from H and CH_3, or the combination of both, and independently thereof,
R``` is selected from H and CH_3, or the combination of both, and wherein ñ is a real number in the range of from 4 to 180.

10. The polymer matrix according to any of claims 8 and 9, wherein R is H.

11. The polymer matrix according to any of claims 8 and 9, wherein R is CH_3.

12. The polymer matrix according to any of claims 8 and 9, wherein R is CH_2OH.

13. The polymer matrix according to any of claims 8 and 9, wherein R is C_2H_5OH

14. The polymer matrix according to any of claims 8 and 9, wherein R is n-C_3H_7

15. The polymer matrix according to any of claims 8 and 9, wherein R is n-C_3H_7

16. The polymer matrix according to any of claims 8 and 9, wherein R is n-C_4H_9

17. The polymer matrix according to any of claims 8 and 9, wherein R is i-C_4H_9

18. The polymer matrix according to any of claims 8 to 17, wherein R` is selected from H, CH_3, i-C_3H_7, n-C_3H_7, i-C_4H_9 and n-C_4H_9.

19. The polymer matrix according to any of claims 8 to 17, wherein R` is H

20. The polymer matrix according to any of claims 8 to 17, wherein R` is CH_3

21. The polymer matrix according to any of claims 8 to 17, wherein R` is n-C_3H_7

22. The polymer matrix according to any of claims 8 to 17, wherein R` is i-C_3H_7

23. The polymer matrix according to any of claims 8 to 17, wherein R` is n-C_4H_9

24. The polymer matrix according to any of claims 8 to 17, whereIn R` is i-C_4H_9

25. The polymer matrix according to any of claims 8 to 24, wherein R`` is H or CH_3

26. The polymer matrix according to any of claims 8 to 24, wherein R`` is H

27. The polymer matrix according to any of claims 8 to 24, wherein R`` is CH_3

28. The polymer matrix according to any of claims 8 to 27, wherein R``` is H or CH_3

29. The polymer matrix according to any of claims 8 to 27, wherein R``` is H.

30. The polymer matrix according to any of claims 8 to 27, wherein R''' is CH_3

31. The polymer matrix according to any of claims 8 to 30, wherein Z is selected from H and CH_3 and C_2H_5, including any combination thereof.

32. The polymer matrix according to any of claims 8 to 30, wherein Z is H

33. The polymer matrix according to any of claims 8 to 30, wherein Z is CH_3

34. The polymer matrix according to any of claims 8 to 30, wherein Z is C_2H_5

35. The polymer matrix according to claim 8, wherein ñ is a real number in the range of from 3 to 800, for example from 3 to 600, such as from 3 to 400, for example from 3 to 300, for example from 3 to 200, such as from 3 to 100, for example from 3 to 90, such as from 3 to 80, for example from 3 to 70, such as from 3 to 60, for example from 3 to 50, such as from 3 to 45, for example from 3 to 40, such as from 3 to 30, for example from 3 to 25.

36. The polymer matrix according to any of the previous claims, wherein the number average molecular weight (M_n) is in the range of from 200 to 60000, such as from 200 to 45000, for example from 200 to 30000, such as from 200 to 25000, for example from 200 to 20000, such as from 200 to 15000, for example from 200 to 10000, such as from 200 to 8000, for example from 200 to 6000, such as from 200 to 5000, for example from 200 to 4500, such as from 200 to 4000, for example from 200 to 3500, such as from 200 to 3000, for example from 200 to 2500, such as from 200 to 2400, for example from 200 to 2300, such as from 200 to 2200, for example from 200 to 2100, such as from 200 to 2000, for example from 200 to 1900, such as from 200 to 1800, for example from 200 to 1700, such as from 200 to 1600, for example from 200 to 1500, such as from 200 to 1400, for example from 200 to 1300, such as from 200 to 1200, for example from 200 to 1100, such as from 200 to 1000, for example from 200 to 900, such as from 200 to 800, for example from 200 to 700, such as from 200 to 600, for example from 400 to 3000, such as from 400 to 2500, for example from 400 to 2000, such as from 400 to 1800, for example from 400 to 1600, such as

from 400 to 1400, for example from 400 to 1200, such as from 400 to 1000, for example from 400 to 800, such as from 400 to 600, for example from 600 to 3000, such as from 600 to 2500, for example from 600 to 2000, such as from 600 to 1800, for example from 600 to 1600, such as from 600 to 1400, for example from 600 to 1200, such as from 600 to 1000, for example from 600 to 800, such as from 800 to 3000, for example from 800 to 2500, such as from 800 to 2000, for example from 800 to 1800, such as from 800 to 1600, for example from 800 to 1400, such as from 800 to 1200, for example from 800 to 1000, such as from 1000 to 3000, for example from 1000 to 2500, such as from 1000 to 2000, for example from 1000 to 1800, such as from 1000 to 1600, for example from 1000 to 1400, such as from 1000 to 1200.

37. The polymer matrix according to any of the previous claims, wherein the amine group loading capacity is in the range of from 0.01 to 14 mmol/gram, such as from 0.01 to 13 mmol/gram, for example from 0.01 to 12 mmol/gram, for example from 0.01 to 11 mmol/gram, such as from 0.01 to 10 mmol/gram, for example from 0.01 to 9 mmol/gram, such as from 0.01 to 8 mmol/gram, for example from 0.01 to 7 mmol/gram, such as from 0.01 to 6 mmol/gram, for example from 0.01 to 5 mmol/gram, such as from 0.01 to 4 mmol/gram, for example from 0.01 to 3 mmol/gram, such as from 0.01 to 2 mmol/gram, for example from 0.01 to 1 mmol/gram, such as from 0.01 to 0.5 mmol/gram, for example from 0.01 to 0.4 mmol/gram, such as from 0.02 to 2 mmol/gram, for example from 0.04 to 2 mmol/gram, such as from 0.06 to 2 mmol/gram, for example from 0.08 to 2 mmol/gram, such as from 0.1 to 13 mmol/gram, for example from 0.1 to 12 mmol/gram, such as from 0.1 to 11 mmol/gram, for example from 0.1 to 10 mmol/gram, such as from 0.1 to 5 mmol/gram, for example from 0.1 to 4 mmol/gram, such as from 0.1 to 3 mmol/gram, for example from 0.1 to 2 mmol/gram, such as from 0.1 to 1.5 mmol/gram, for example from 0.1 to 1 mmol/gram, such as from 0.1 to 0.8 mmol/gram, for example from 0.1 to 0.6 mmol/gram, such as from 0.1 to 0.5 mmol/gram, for example from 0.1 to 0.4 mmol/gram, such as from 0.1 to 0.3 mmol/gram, for example from 0.1 to 0.2 mmol/gram, such as from 0.2 to 2 mmol/gram, for example from 0.4 to 2 mmol/gram, such as from 0.6 to 2 mmol/gram, for example from 0.8 to 2 mmol/gram, such as from 0.9 to 2 mmol/gram, for example from 1.5 to 2 mmol/gram, such as from 0.4 to 1.3 mmol/gram, for example from 0.6 to 1.3

mmol/gram, such as from 0.8 to 1.3 mmol/gram, for example from 1 to 2 mmol/gram, such as from 1.2 to 2 mmol/gram, for example from 1.4 to 2 mmol/gram, such as from 1.6 to 2 mmol/gram, for example from 1.8 to 2 mmol/gram, such as from 0.01 to 0.05 mmol/gram, for example from 0.05 to 0.1 mmol/gram, such as from 0.1 to 0.2 mmol/gram, for example from 0.2 to 0.4 mmol/gram, such as from 0.4 to 0.6 mmol/gram, for example from 0.6 to 0.8 mmol/gram, such as from 0.8 to 1 mmol/gram, such as from 1 to 1.2 mmol/gram, for example from 1.2 to 1.4 mmol/gram, such as from 1.4 to 1.6 mmol/gram, for example from 1.6 to 1.8 mmol/gram.

38. The polymer matrix according to any of the previous claims, wherein the swelling volume of the matrix in an aqueous liquid, including water, is in the range of from 1 ml/gram to preferably less than 32 ml/gram, such as from 1 ml/gram to 24 ml/gram, for example from 1 ml/gram to 20 ml/gram, such as from 1 ml/gram to 18 ml/gram, for example from 1 ml/gram to 16 ml/gram, such as from 1 ml/gram to 14 ml/gram, for example from 1 ml/gram to 12 ml/gram, such as from 1 ml/gram to 10 ml/gram, for example from 1 ml/gram to 9 ml/gram, such as from 1 ml/gram to 8 ml/gram, for example from 1 ml/gram to 7 ml/gram, such as from 1 ml/gram to 6 ml/gram, for example from 1 ml/gram to 5 ml/gram, such as from 1 ml/gram to 4 ml/gram, for example from 1 ml/gram to 3 ml/gram, such as from 1 ml/gram to 2 ml/gram, for example from 4 ml/gram to 20 ml/gram, such as from 4 ml/gram to 18 ml/gram, for example from 4 ml/gram to 16 ml/gram, such as from 4 ml/gram to 14 ml/gram, for example from 4 ml/gram to 12 ml/gram, such as from 4 ml/gram to 10 ml/gram, for example from 4 ml/gram to 8 ml/gram, such as from 4 ml/gram to 6 ml/gram, for example from 6 ml/gram to 20 ml/gram, such as from 6 ml/gram to 18 ml/gram, for example from 6 ml/gram to 16 ml/gram, such as from 6 ml/gram to 14 ml/gram, for example from 6 ml/gram to 12 ml/gram, such as from 6 ml/gram to 10 ml/gram, for example from 6 ml/gram to 8 ml/gram, such as from 8 ml/gram to 20 ml/gram, for example from 8 ml/gram to 16 ml/gram, such as from 8 ml/gram to 12 ml/gram, for example from 2 ml/gram to 4 ml/gram, such as from 8 ml/gram to 10 ml/gram, for example from 10 ml/gram to 12 ml/gram, such as from 12 ml/gram to 14 ml/gram, for example from 14 ml/gram to 16 ml/gram, such as from 16 ml/gram to 18 ml/gram, for example from 18 ml/gram to 20 ml/gram.

39. The polymer matrix according to any of the previous claims, wherein the ratio R between i) the amine group loading capacity and ii) the swelling volume of the matrix in an aqueous liquid, including water, is in the range of from 10^{-4} to 0.5, such as from 10^{-4} to 0.4, for example from 10^{-4} to 0.3, such as from 10^{-4} to 0.2, for example from 10^{-4} to 0.1, such as from 10^{-4} to 0.09, for example from 10^{-4} to 0.08, such as from 10^{-4} to 0.07, for example from 10^{-4} to 0.06, such as from 10^{-4} to 0.05, for example from 10^{-4} to 0.04, such as from 10^{-4} to 0.03, for example from 10^{-4} to 0.02, such as from 10^{-4} to 0.01, for example from 10^{-4} to 0.009, such as from 10^{-4} to 0.005, for example from 10^{-3} to 0.5, such as from 10^{-3} to 0.4, for example from 10^{-3} to 0.3, such as from 10^{-3} to 0.2, for example from 10^{-3} to 0.1, such as from 10^{-3} to 0.09, for example from 10^{-3} to 0.08, such as from 10^{-3} to 0.06, for example from 10^{-3} to 0.04, such as from 0.01 to 0.5, for example from 0.1 to 0.5, such as from 0.01 to 0.4, for example from 0.02 to 0.04, such as from 0.04 to 0.08, for example from 0.05 to 0.5, such as from 0.08 to 0.5.

40. The polymer matrix according to any of the previous claims, wherein said matrix is beaded and has an essentially spherical form.

41. The polymer matrix according to claim 40 having a diameter in the range of from 0.1 μm to preferably less than 3000 μm, preferably a diameter in the range of between 10 μm and 1000 μm.

42. The polymer matrix according to any of claims 40 and 41, formed by polymerization of droplets in an inert phase, such as unreactive oil, for example paraffin oil.

43. The polymer matrix according to any of claims 1 to 39 formed by bulk polymerization followed by granulation.

44. A method for preparing the beaded polymer matrix according to any of claims 1 to 42, said method comprising the step of

a) providing a macromonomer comprising a bisamino poly(alkylene)glycol functionalized with at least one fragment comprising a conjugated vinyl group,

b) mixing the conjugated vinyl macromonomer with acrylic amide derivatives,

c) copolymerizing the vinyl groups of said macromonomers using radical initiators or ionic initiators,

d) forming a beaded, cross-linked polymer matrix comprising a plurality of amide functionalities,

e) reducing at least 50% of the amide functionalities, and

f) obtaining a beaded polymer matrix wherein the majority of the amide functionalities are reduced to primary and secondary amine functionalities.

45. The method of claim 44, wherein the reduction of the amide functionalities in step e) is performed using a reducing agent selected from the group consisting of borane, arsenic trioxide in aqueous alcoholic HCl, antimony pentoxide in aqueous alcoholic HCl, $LiAlH_4$, H_2O_2, $BF_3{\cdot}Et_2O$ in the presence of sodium borate, lithium tri-(tert)-butoxyaluminium hydride ($LiAlH(OtBu)_3$), DIBAL-H, $NaBH_4$, $NaBH_3CN$ and NaH.

46. Use of the polymer matrix according to any of claims 1 to 43 for a support for the synthesis of an organic molecule.

47. Use of the polymer matrix according to any of claims 1 to 43, for scavenging excess acyl compounds from a composition comprising a mixture of molecular entities.

48. Use of the polymer matrix according to any of claims 1 to 43, for scavenging excess carbonyl compounds from a composition comprising a mixture of molecular entities.

49. Use of the polymer matrix according to any of claims 1 to 43, for solid phase enzyme reactions.

50. Use of the polymer matrix according to any of claims 1 to 43, as a support for the chemical and/or enzymatic synthesis of a peptide, a protein, a DNA, a RNA or an oligosaccharide.

51. Use of the polymer matrix according to any of claims 1 to 43, for protein immobilization.

52. Use of the polymer matrix according to any of claims 1 to 43, for chromatographic separation or purification.

53. The use of claim 52, wherein the chromatographic separation or purification comprises at least one step employing affinity purification.

54. Use of the polymer matrix according to any of claims 1 to 43, as a support for combinatorial chemistry.

55. Use of a partially acryloylated bisamino poly(alkylene)glycol in the preparation of the beaded, cross-linked polymer matrix according to any of claims 1 to 43.

56. The use of claim 55, wherein the preparation comprises the step of inverse suspension polymerization.

57. A composition comprising a plurality of cross-linked polymer beads according to any of claims 1 to 42.

58. The composition according to claim 57, wherein the average diameter of the beads is in the range of from 0.1 μm to preferably less than 3000 μm.

59. A functional surface comprising the polymer matrix according to any of claims 1 to 43, and attached thereto at least one functional moiety.

60. The functional surface according to claim 59, wherein the functional moiety is a bioactive species preferably selected from a scaffold moiety comprising at least one site for functionalization, a RNA moiety, a DNA moiety , a peptide moiety, or an amino acid residue.

61. The functional surface according to claim 59 or 60, wherein said surface is attached to a solid support.

62. The functional surface according to claim 59 to 61, wherein the said surface is planar, tubular, spherical or a porous material.

63. The functional surface according to claim 60 to 62, wherein said surface further comprises a linker residue, preferably a linker residue comprising functional groups consisting of amino, alkylamino, hydroxy, carboxyl, mercapto, sulfeno, sulfino, sulfo, and derivatives of these.

64. A method for preparing a functional surface according to any of claims 59 to 63, said method comprising the steps of

a) providing a macromonomer comprising a bisamino poly(alkylene)glycol functionalized with at least one fragment comprising a vinyl group,

b) mixing the conjugated vinyl macromonomer with acrylic amide derivatives

c) polymerizing the vinyl groups of said macromonomers using radical initiators or ionic initiators,

d) forming a beaded, cross-linked polymer matrix comprising a plurality of amide functionalities,

e) reducing the amide functionalities and obtaining a beaded polymer matrix wherein the majority of the amide functionalities are reduced to primary and secondary amine functionalities, and

f) contacting the beaded polymer matrix obtained in step e) with at least one functional moiety, and

g) obtaining the functional surface.

65. A method for targeting a functional moiety attached to a functional surface, said method comprising the steps of

a) providing a functional surface according to any of claims 59 to 63, and

b) targeting said functional moiety with at least one targeting species having a noncovalent affinity, for said functional moiety, or

c) targeting said functional moiety with at least one targeting species forming a covalent bond with the said functional moiety.

66. A method for identifying and/or purifying a targeting species having an affinity for a functional moiety, said method comprising the steps of

a) providing a functional surface according to any of claims 59 to 63, and

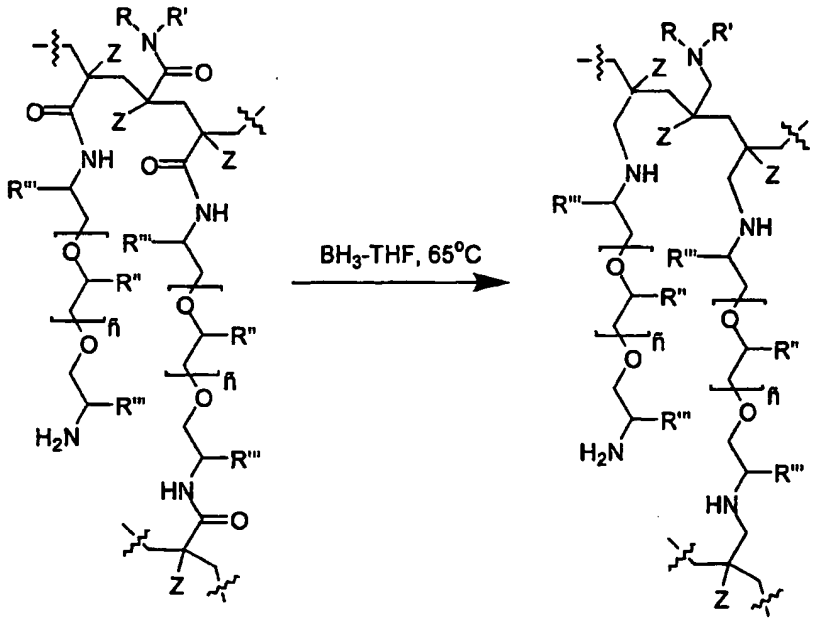
b) targeting said functional moiety with at least one targeting species having an affinity for said functional moiety, and

c) identifying and/or purifying the at least one targeting species having an affinity for said functional moiety.

67. A targeting species identified and/or purified by the method of claim 66.

68. A method for therapy of a human or animal body, said method comprising the step of administering to said human or animal body a targeting species according to claim 67 in a pharmaceutical effective amount.

Fig. 1



Z = H or CH_3 or C_2H_5
R = H or CH_3 or CH_2OH or C_2H_5OH or i-C_3H_7 or n-C_3H_7 or i-C_4H_9 or n-C_4H_9
R' = H or CH_3 or i-C_3H_7 or n-C_3H_7 or i-C_4H_9 or n-C_4H_9
R" = H or CH_3
R"' = H or CH_3

Fig. 2

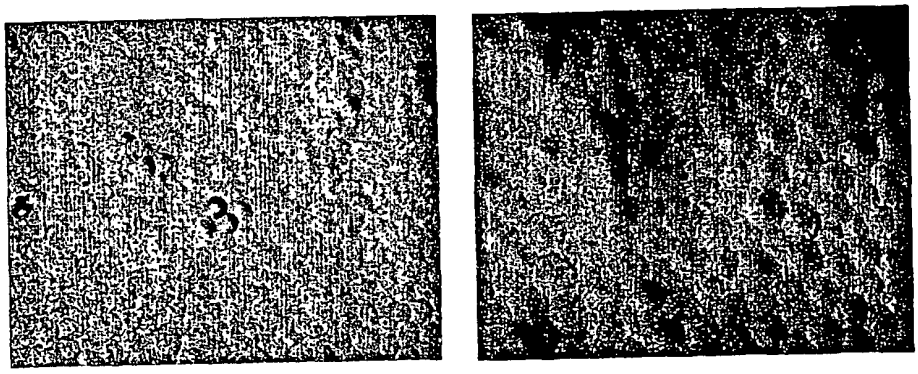


Fig. 3

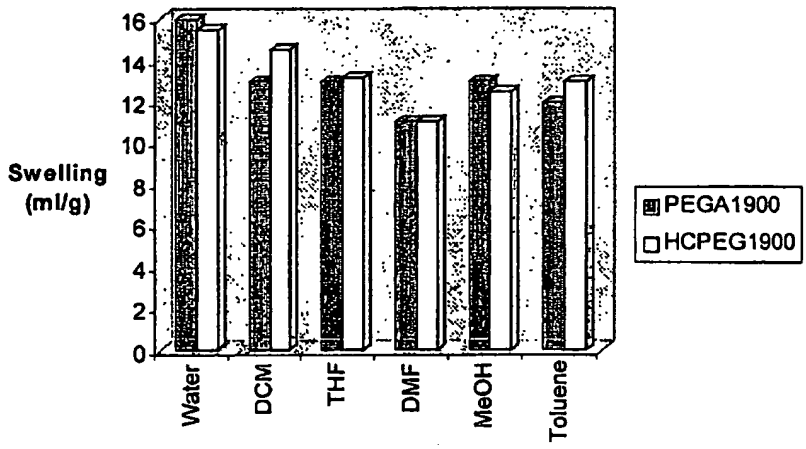

Swelling (ml/g)
16
14
12
10
8
6
4
2
0
Water
DCM
THF
DMF
MeOH
Toluene
PEGA1900
HCPEG1900

Fig. 4

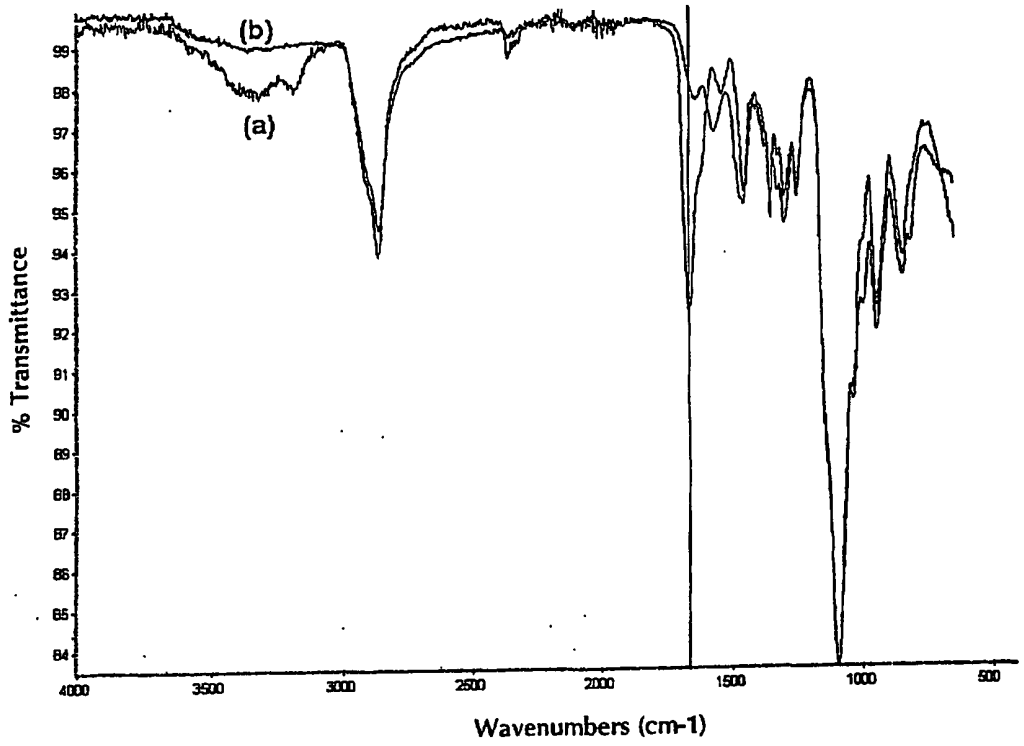
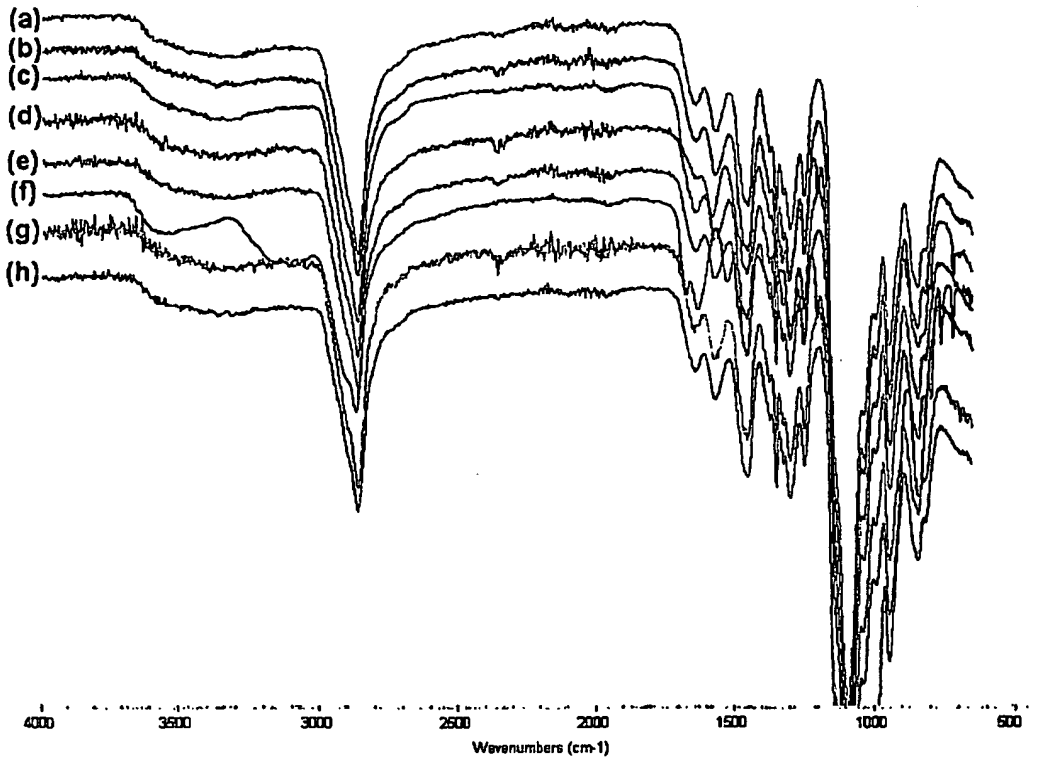
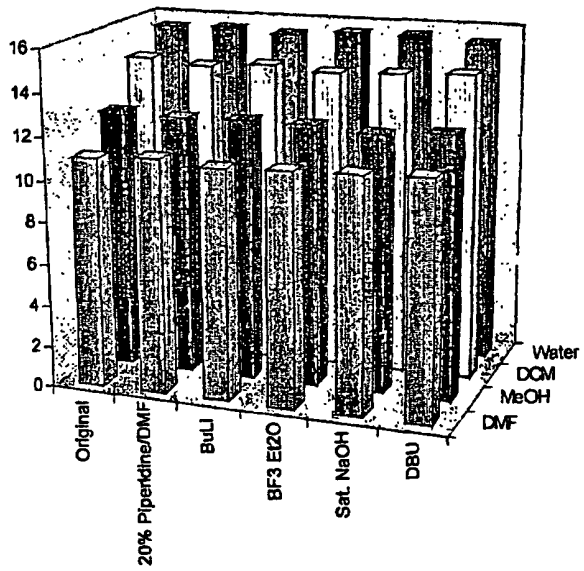

(b)
(a)
% Transmittance
99
98
97
96
95
94
93
92
91
90
89
88
87
86
85
84
4000
3500
3000
2500
2000
1500
1000
500
Wavenumbers (cm-1)

Fig. 5



Wavenumbers (cm-1)

Fig. 6


16
14
12
10
8
6
4
2
0
Original
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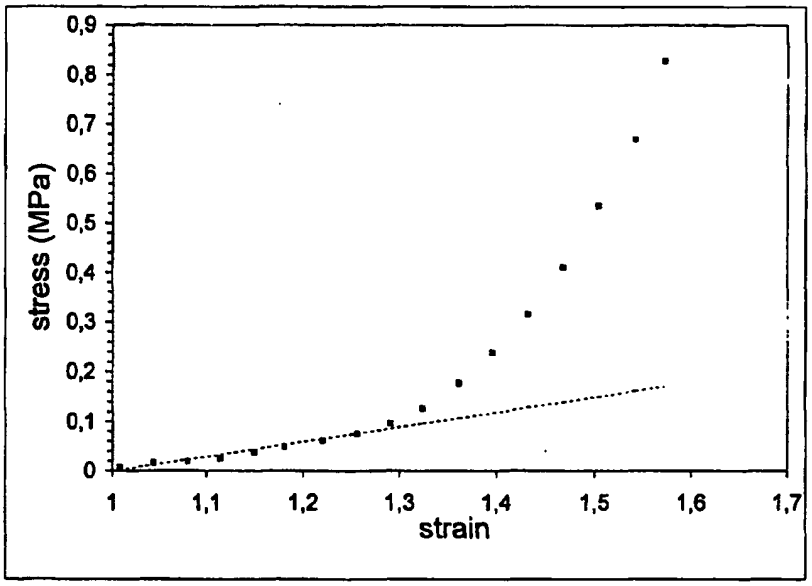
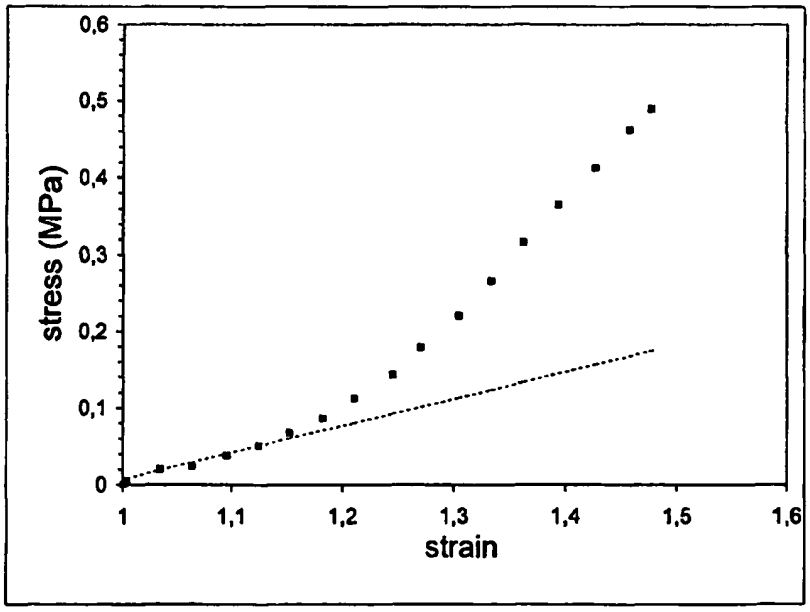
BuLi
BF3 Et2O
Sat. NaOH
DBU
Water
DCM
MeOH
DMF

Fig. 7

A


stress (MPa)
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
1
1,1
1,2
1,3
1,4
1,5
1,6
1,7
strain

B


stress (MPa)
0,6
0,5
0,4
0,3
0,2
0,1
0
1
1,1
1,2
1,3
1,4
1,5
1,6
strain

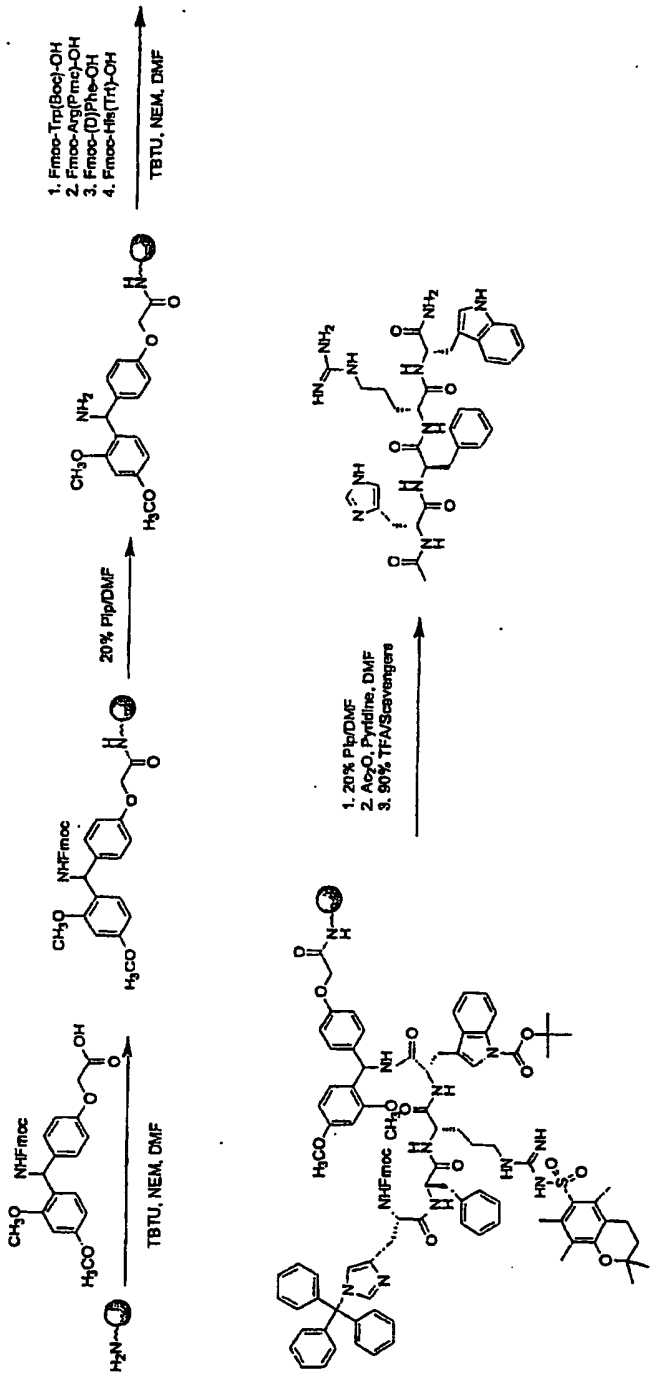

TBTU, NEM, DMF
20% Pip/DMF
1. Fmoc-Trp(Boc)-OH
2. Fmoc-Arg(Pmc)-OH
3. Fmoc-(D)Phe-OH
4. Fmoc-His(Trt)-OH
TBTU, NEM, DMF
1. 20% Pip/DMF
2. Ac2O, Pyridine, DMF
3. 90% TFA/Scavengers

Fig. 8

Fig. 9

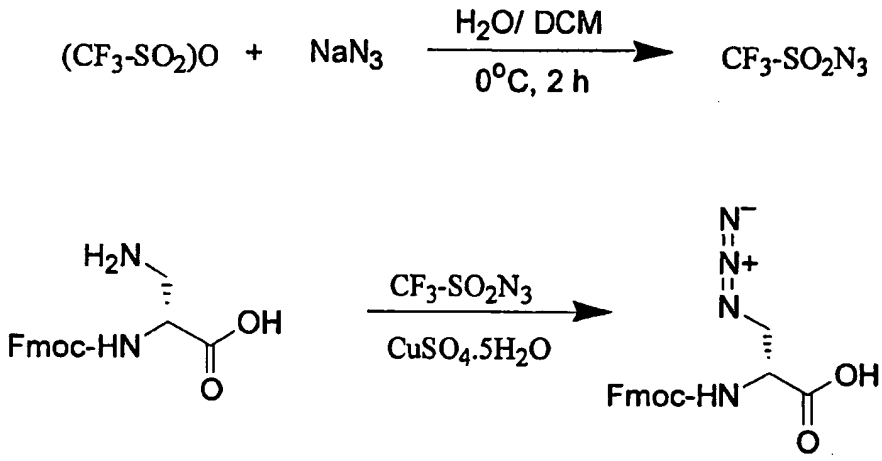

(CF3-SO2)O + NaN3
H2O/ DCM
0°C, 2 h
CF3-SO2N3
H2N
Fmoc-HN
OH
O
CF3-SO2N3
CuSO4.5H2O
N−
N+
N
Fmoc-HN
OH
O

Fig. 10

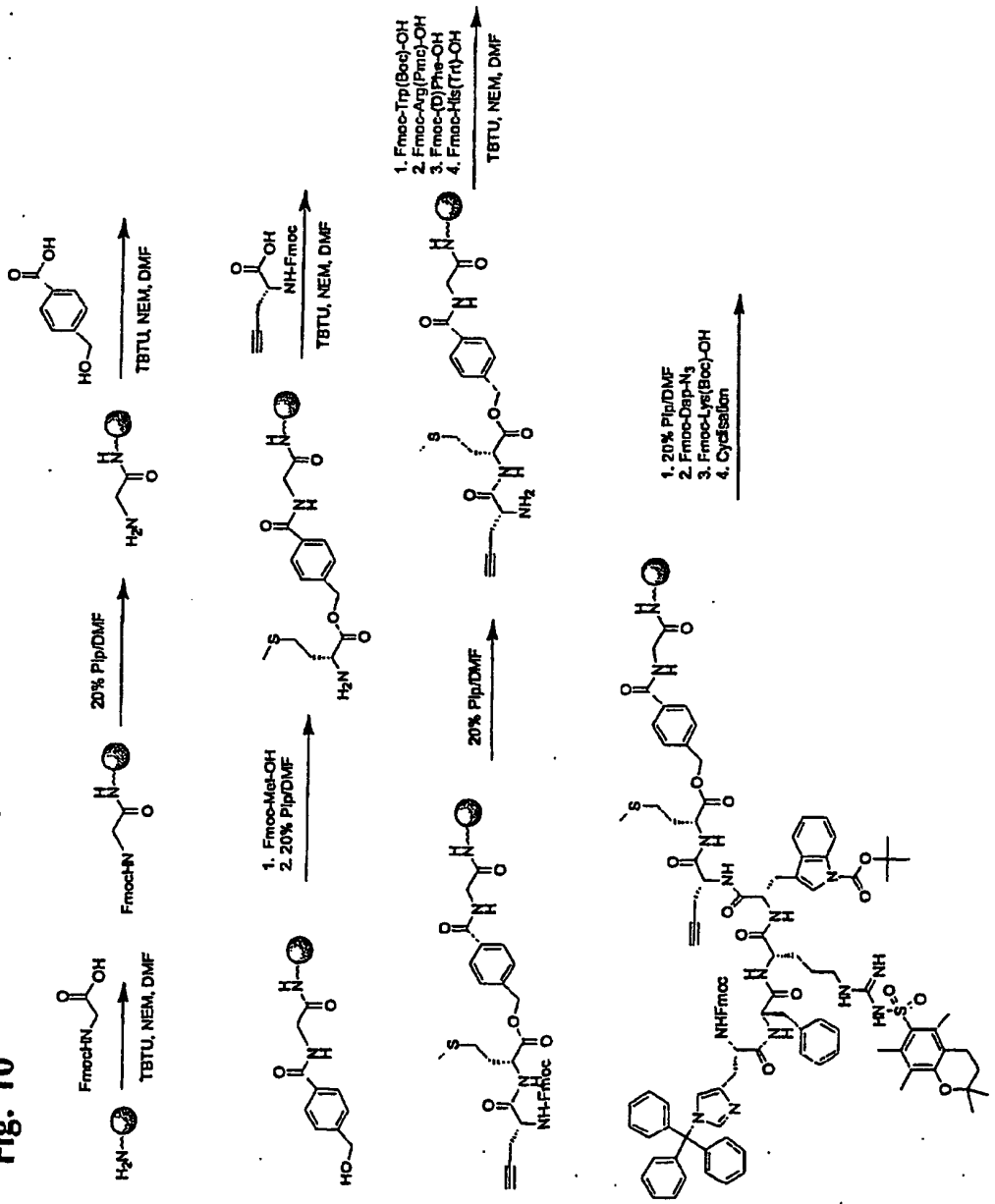

TBTU, NEM, DMF
20% Pip/DMF
1. Fmoc-Met-OH
2. 20% Pip/DMF
TBTU, NEM, DMF
1. Fmoc-Trp(Boc)-OH
2. Fmoc-Arg(Pmc)-OH
3. Fmoc-(D)Phe-OH
4. Fmoc-His(Trt)-OH
TBTU, NEM, DMF
20% Pip/DMF
1. 20% Pip/DMF
2. Fmoc-Dap-N3
3. Fmoc-Lys(Boc)-OH
4. Cyclisation

Fig. 10 (continued)

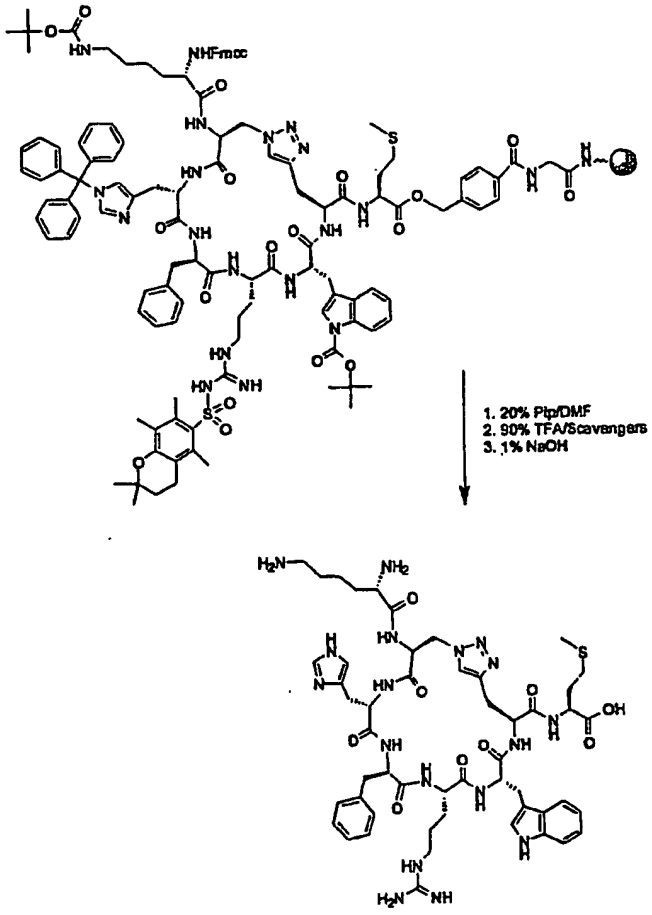


Fig. 11

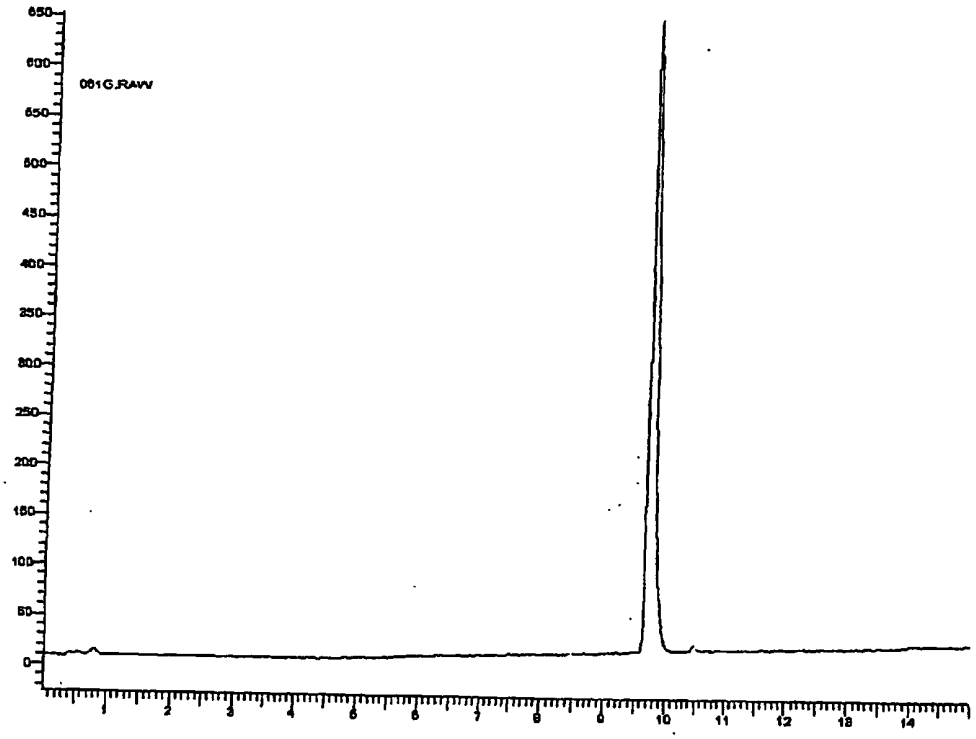

061G.RAW
650
600
550
500
450
400
350
300
250
200
150
100
50
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14

Fig. 12

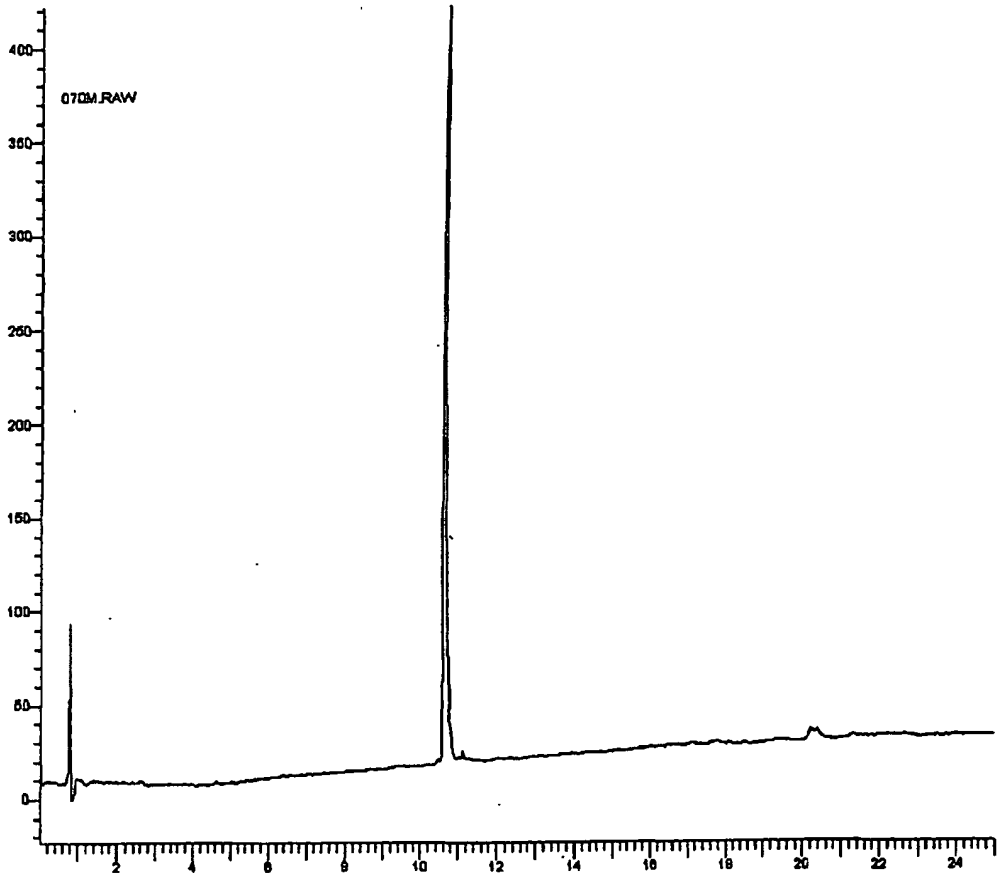
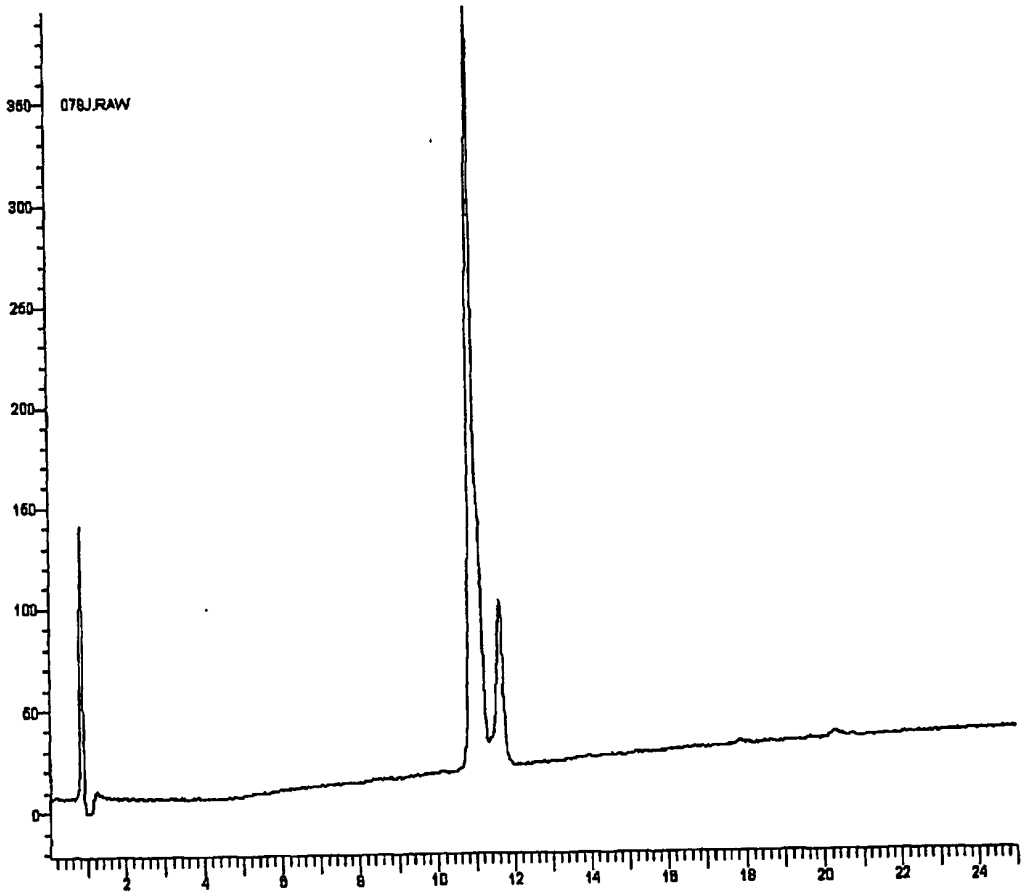

070M.RAW
400
350
300
250
200
150
100
50
0
2
4
6
8
10
12
14
16
18
20
22
24

Fig. 13



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
29 December 2004 (29.12.2004)

PCT

(10) International Publication Number
WO 2004/113389 A3

(51) International Patent Classification[7]: C08F 8/04, C08G 65/32, C08L 71/02

(21) International Application Number: PCT/DK2004/000461

(22) International Filing Date: 28 June 2004 (28.06.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PA 2003 00963 26 June 2003 (26.06.2003) DK
60/482,452 26 June 2003 (26.06.2003) US

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(72) Inventors; and

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(74) Agent: HØIBERG A/S; St. Kongensgade 59A, DK-1264 Copenhagen K (DK).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

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Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for all designations*

— *of inventorship (Rule 4.17(iv)) for US only*

Published:

— *with international search report*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report: 27 January 2005

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: HIGH CAPACITY POLY(ALKYLENE)GLYCOL BASED AMINO POLYMERS

(57) Abstract: The present invention relates to a cross-linked and beaded, stable and high loading capacity polymer matrix for affinity chromatography applications and for solid phase synthesis. The polymer matrix can be obtained by a method comprising the steps of providing a plurality of macromonomers each comprising a poly(oxalkylene) chain terminated with an acylamide functional group, polymerising said macromonomers using a free radical initiator or an ionic initiator, optionally with the addition of copolymerizing agents, and converting in the beaded polymer matrix at least 50% of the amide groups to amine functional groups by reduction of the amide groups with a suitable reducing agent.

WO 2004/113389 A3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK2004/000461

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C08F8/04 C08G65/32 C08L71/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C98L C08L C08G C08F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4 745 134 A (OSEI-GYIMAH PETER) 17 May 1988 (1988-05-17) claims 1,2; example 1	1-68
A	WO 03/031489 A (MIRANDA LESLIE P ; MELDAL MORTEN (DK); CARLSBERG AS (DK)) 17 April 2003 (2003-04-17) claim 1; figure 1	1-68
A	WO 00/18823 A (BUCHARDT JENS ; MELDAL MORTEN (DK); CARLSBERG AS (DK); RADEMANN JOERG) 6 April 2000 (2000-04-06) claims 1,12	1-68
A	GB 1 558 491 A (BAYER AG) 3 January 1980 (1980-01-03) claims 1,4	1-68

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 December 2004	16/12/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	O'Sullivan, T

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK2004/000461

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99/01469 A (DAVIS STANLEY STEWART ; UNIV NOTTINGHAM (GB); GARNETT MARTIN CHARLES () 14 January 1999 (1999-01-14) example 1b	1-68

Form PCT/ISA/210 (continuation of second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

ternational application No.
PCT/DK2004/000461

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 68
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 68 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.